의학박사 학위논문

Cardia cancer is different from non-cardia cancer in terms of local and systemic immune responses

비분문부암과 다른 국소-전신 면역반응을 보이는 분문부암

2018 년 2 월

서울대학교 대학원

의학과 박사과정

김 형 일

A thesis of the Degree of Doctor of Philosophy

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by

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A thesis submitted to the Department of surgery in partial fulfillment of the requirement of the Degree of Doctor of Philosophy in surgery at Seoul National University College of Medicine

February 2018

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비분문부암과 다른 국소-전신 면역반응을 보이는 분문부암

지도교수 이 혁 준

이 논문을 의학박사 학위논문으로 제출함

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ABSTRACT

Introduction: This study sought to investigate the prognostic significance of tumor infiltrating lymphocytes (TILs) in respect to the prognostic nutritional index (PNI), neutrophil-to-lymphocyte ratio (NLR), and the topographic location of gastric tumors.

Methods: Retrospective data from a prospectively maintained database of gastric cancer patients who underwent gastrectomy from January 2001 to December 2010 at a single center were retrieved. The distribution and prognostic significance of a subset of TILs using immunohistochemical staining for CD3, CD4, CD8, Foxp3, and granzyme B in 416 gastric cancer patients were evaluated. The PNI was calculated using preoperative laboratory values of 7781 gastric cancer patients. TILs and PNI were analyzed according to topographic location.

Results: Gastric cancers in the cardia, compared to other locations, were associated with significantly lower CD8 and higher Foxp3 and granzyme B counts, without significant differences in PNI or NLR values. In cardia-localized cancer, multivariate analysis for clinicopathological and immunological factors revealed that lymph node metastasis and a high Foxp3/CD4 ratio were independent poor prognostic factors for overall survival. In non-cardia cancer, total gastrectomy, advanced T-classification, lymph node metastasis, low Foxp3, and low PNI were all poor prognostic factors.

Conclusions: The distribution and prognostic impact of TILs and PNIs varied according to the longitudinal location of the cancer. Regulatory T

lymphocytes were an unfavorable prognostic factor in cardia cancer and a favorable prognostic factor in non-cardia cancer.

Keywords: Tumor infiltrating lymphocytes; regulatory T-lymphocytes; prognostic nutritional index; cardia cancer; gastric cancer; prognosis; immune responses

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LIST OF ABBREVIATIONS

TILs: tumor infiltrating lymphocytes

Foxp3: Forkhead/winged helix transcription factor

PNI: prognostic nutritional index

NLR: neutrophil-to-lymphocyte ratio

GZB: granzyme B

OS: overall survival

RFS: relapse-free survival

HR: hazard ratio

OR: odds ratio

95% CI: 95% confidence interval

AUC: area under the curve

iAUC: integrated area under the curve

GENERAL INTRODUCTION

Gastric cancer is the fifth most common cancer and the third leading cause of cancer-related deaths worldwide.(1) Although cancer stage is the best tool for determining prognosis and adjuvant treatment, treatment outcomes can vary between individuals of the same stage. To address this heterogeneity, studies have begun to investigate the interactions of intrinsic tumor cell characteristics, as well as other tumor-associated characteristics, such as tumor microenvironment and host immune status.(2) Herein, I sought to explore the prognostic impact of tumor infiltrating lymphocytes (TILs) as a marker for local immune responses and the prognostic nutritional index (PNI) and neutrophil to lymphocytes ratio (NLR) as markers for systemic immune responses in relationship to the location of tumors.

Tumor infiltrating lymphocytes are considered prognostic factors because they represent the local host anti-tumor immunity of human malignancies, including melanoma, colorectal, ovarian, esophageal, liver, and lung cancers.(3-9) While several studies have demonstrated that pronounced lymphocytic infiltration is associated with better prognosis, (3, 5) regulatory lymphocytes, a subset of TILs, have been shown to adversely affect patient survival.(10) While some reports have found regulatory T cells to hold poor prognostic power,(11-20) others have demonstrated a favorable prognostic impact for regulatory T cells.(21-23) However, the impact of TILs on prognosis has not been approached in the context of clinically relevant information, such as stage(15, 20) and tumor location.(11, 16, 18, 20, 24-27)

Contrary to TILs, which reflect local immune responses of the host, the prognostic nutritional index (PNI) and neutrophil-to-lymphocyte ratio (NLR)

can be used as parameters of systemic immune responses of the host. PNI and NLR can be easily calculated using information obtained during routine preoperative laboratory examination, including albumin levels, lymphocyte counts, and neutrophil counts. While the value of these parameters has been identified in various types of cancer patients,(28-31) only a few reports have evaluated the clinical significance of PNI in gastric cancer,(30, 32) or within the context of cancer TILs.(33-36)

Thus, this study analyzed the prognostic impact of local and systemic immunological parameters in the context of clinicopathological parameters, especially the longitudinal location of tumor. In colorectal cancer, the anatomical site of a tumor has been shown to be an important factor in clinical management.(37, 38) Furthermore, accumulating evidence has shed light on distinct differences in molecular pathway characteristics associated with the anatomical site of a tumor.(39, 40) In other cancers, the anatomical location and functions of individual tumors have been found to reflect different tumor microenvironments, a phenomenon that may be applicable to gastric cancer (Figure 1): Cardia cancers have been found to be associated with gastroesophageal reflux(41, 42) and obesity.(41, 43-45), and in recent analyses, the incidence of cardia cancers has remained stable or increased.(41, 46-48) In comparison, non-cardia cancers have been linked to *Helicobacter pylori* infection,(49) and the incidence of this type of cancer is actually decreasing.(42, 47-49) This study hypothesized that the location of tumors in the gastric tube would be associated with different local and systemic immunological characteristics.

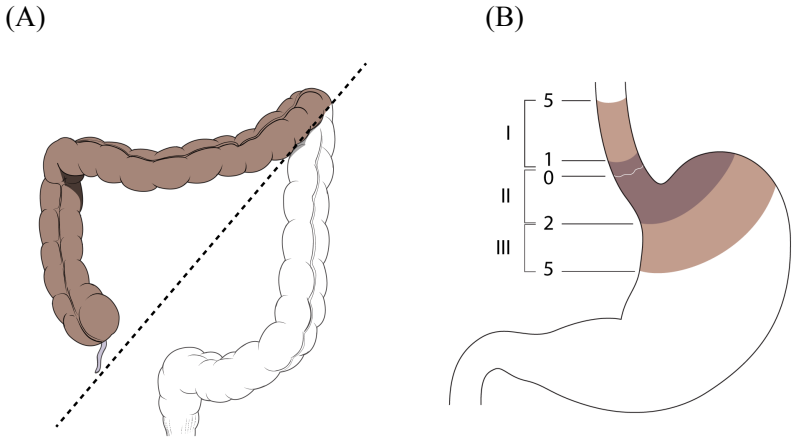

Figure 1. Clinicopathological differences according to tumor location in the colon and stomach

As colon cancers in the right and left colon show different clinicopathologic and molecular characteristics (A), this study assumed that stomach cancer may also exhibit different immune responses in cardia and non-cardia locations (B). Cardia location was classified as Siewert types I, II, and III according to the relative distance from the esophagogastric junction: type I: from 1 cm above to 5 cm above the esophagogastric junction; type II: from 1 cm above to 2 cm below the esophagogastric junction; and type III: from 5 cm below to 2 cm below the esophagogastric junction

CHAPTER 1

Local immune responses in cardia cancer

INTRODUCTION

Gastric cancer in the cardia comprises an increasing proportion of gastric cancer patients with poor prognosis, and is thought to differ pathophysiology from that of distal gastric cancer.(51) Despite considerable investigation into the clinico-pathologic features of cardia cancer, the biological behavior thereof is still unknown.

The interaction of the tumor microenvironment and the immune system plays a crucial role in cancer development and progression.(52) Tumor-infiltrating lymphocytes (TILs) are considered prognostic factors because they represent the local host anti-tumor immunity of human malignancies, including melanoma, colorectal, ovarian, esophageal, liver, and lung cancers.(3-9) While several studies have demonstrated that pronounced lymphocytic infiltration are associated with better prognosis, (3, 5) regulatory lymphocytes, a subset of TILs, have been shown to adversely affect patient survival.(10) This suggests that the types, not the quantity, of TILs are a critical factor affecting the prognosis of cancer patients.

Tumor-infiltrating lymphocytes consist of various antitumor effectors and regulatory subsets. CD8+ T lymphocytes and CD4+ helper T lymphocytes are effector cells and are thought to be associated with favorable prognosis.(6) While CD8+ T cells are the main effectors of antitumor immunity, CD4+ helper T cells induce and maintain CD8+ T cells.(53) On the other hand, regulatory lymphocytes, a subset of T cells that inhibit anti-tumor immune reactions, are known to be associated with unfavorable prognosis.(7-10, 19, 54) The forkhead/winged helix transcription factor (Foxp3) is a unique molecule that distinguishes ‘regulatory cells’ from conventional ‘helper cells’

among the CD4+ helper T cells.(55) Regulatory T cells are known to attenuate host anti-tumor immunity by suppressing T-cell proliferation, antigen presentation, and cytokine production.(56) As the tumor progresses and becomes established in the host, populations of TILs are skewed to favor regulatory T cells over helper CD4+ T cells.(53)

In this study, the prognostic significance of TILs, as well as recurrence patterns, in gastric cardia cancer patients who underwent curative resection were investigated. Subsets of TILs were evaluated using immunohistochemical staining with monoclonal antibodies against CD3, CD4, CD8, Foxp3, and granzyme B in the resected tumor specimens.

MATERIALS AND METHODS

1. Patients

Retrospective analysis of a prospectively collected database of gastric cancer patients was performed. From January 1, 2000 to December 31, 2006, 197 consecutive patients with gastric cardia cancer underwent curative resection at Severance Hospital, Yonsei University College of Medicine. Follow-up of the patients ended on December 31, 2009, with a median follow-up time of 45 months. Inclusion criteria were gastric adenocarcinoma without distant metastasis and curative surgical resection with or without adjuvant chemotherapy (**Figure 1**). Excluded patients comprised those with neoadjuvant chemotherapy or radiation therapy, history of other primary cancers,

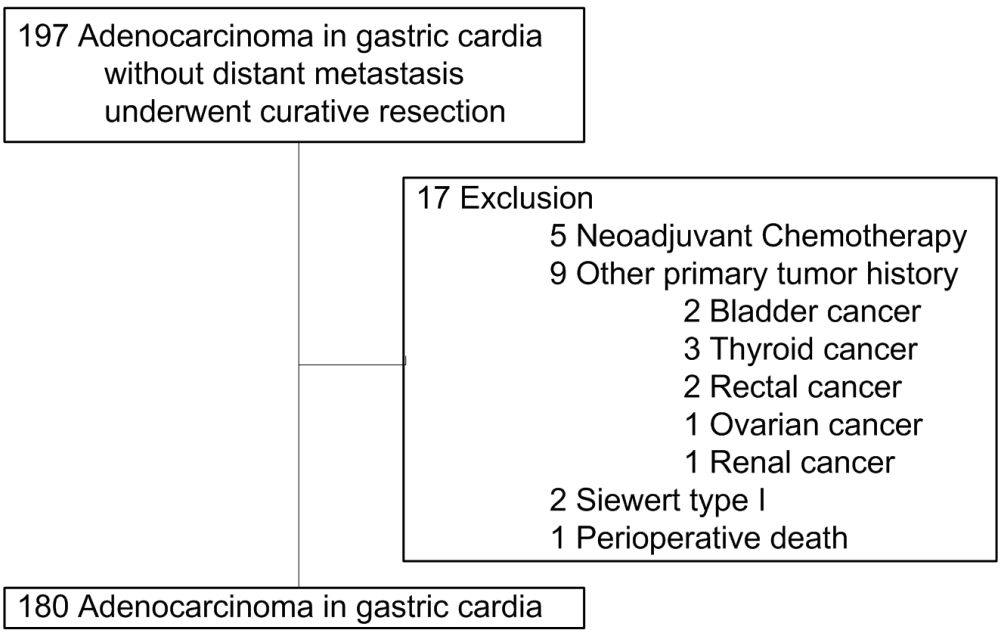

Figure 1. Flow diagram

Diagram showing the flow for selecting the patients in this study

Siewert type I cardia cancer, and death within 30 days of surgery. Seventeen patients were excluded from the study for these reasons. Finally, the demographics, histopathologic, and survival data of 180 patients were analyzed. Staging and histologic grade were recorded according to the American Joint Committee on Cancer 7th Edition.(57) This study was approved by the Yonsei Institutional Review Board.

2. Immunohistochemistry of tumor-infiltrating T-lymphocyte subsets

Immunohistochemical staining was performed on paraffin-embedded cardia cancer tissue sections that had been serially sectioned at 4-μm thickness after using hematoxylin and eosin staining as a guide (**Figure 2**). The sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was performed in citrate buffer in a microwave. Endogenous peroxidase activity was blocked by incubating specimens in 3% hydrogen peroxide in methanol for 5 minutes. The sections were incubated for 60 minutes at room temperature with primary monoclonal antibodies: CD3 (1:100, Labvision Corporation, Fremont, CA, USA), CD4 (1:100, Novocastra, Newcastleupon Tyne, UK), CD8 (1:100, Novocastra), Foxp3 (1:100, Abcam, ab20034, Cambridge, UK), and granzyme B (1:100, Labvision Corporation) to identify total T lymphocytes, helper T lymphocytes, cytotoxic T lymphocytes, regulatory T cells, and activated cytotoxic T lymphocytes, respectively. Incubation in horseradish peroxidase-conjugated secondary antibody was subsequently performed, followed by development with diaminobenzidine and counterstaining with hematoxylin. Between solution changes, the slides were rinsed twice in 0.05 mol/L Tris-buffered saline with 0.2% Tween-20. Normal human tonsil tissue was used

as a positive control. A negative control for immunostaining was prepared by incubating tissue sections without primary antibody.

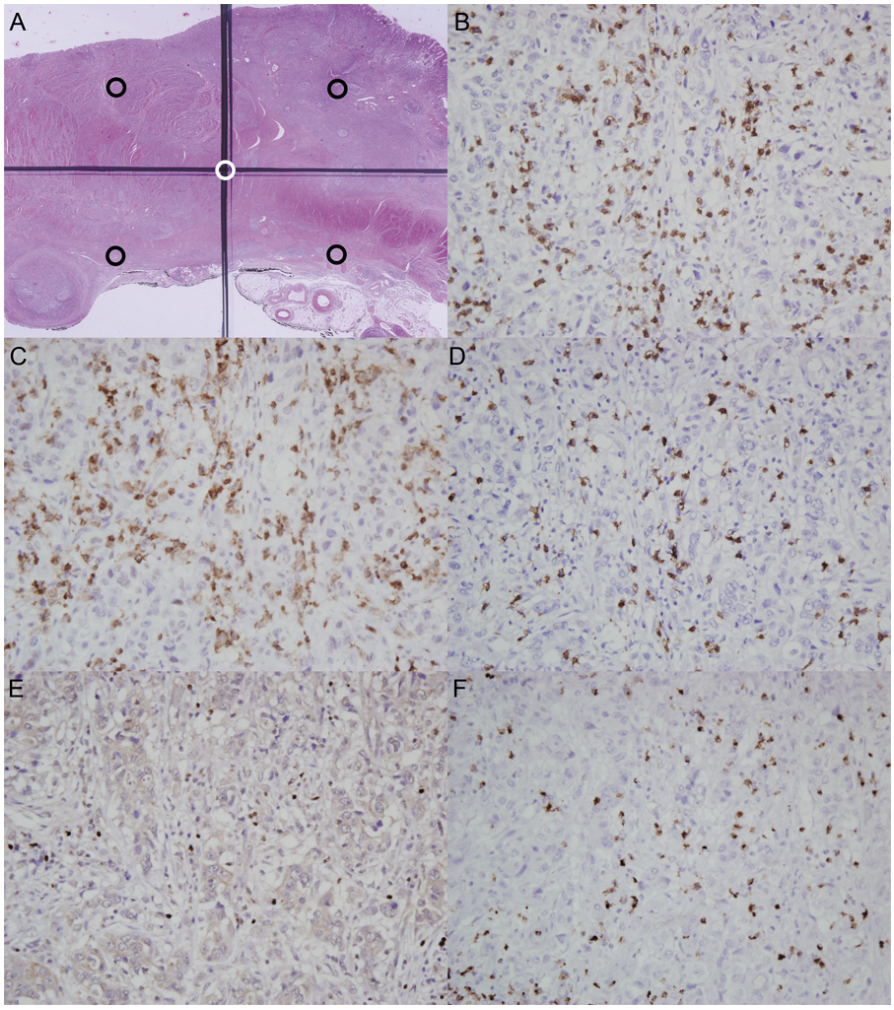

Figure 2. Hematoxylin, eosin, and immunohistochemical detection of tumor-infiltrating T-lymphocytes

Hematoxylin and eosin staining of sections from a paraffin block (A) to confirm the quality of tissue and areas of immunohistochemical staining. The center of the paraffin block and the center of four quadrants were selected for counting. Immunohistochemical detection of (B) CD3 (T lymphocytes), (C) CD4 (helper T lymphocytes), (D) CD8 (cytotoxic T lymphocytes), (E) Foxp3 (regulatory T lymphocytes), and (F) granzyme B (activated cytotoxic T lymphocytes) in consecutive sections are shown (original magnification, ×400).

3. Quantification of tumor-infiltrating T-lymphocyte subsets

An experienced pathologist who was blinded to the patient data reviewed the slides. Five high-power fields (×400) from each slide were selected for immunohistochemical evaluation as follows: The tumor portion from each slide was divided into four quadrants, and the center of each quadrant and the center of the tumor was evaluated (Figure 2A). Areas of the tumor with necrosis or hemorrhage were avoided. The mean numbers of positively stained cells per high-power fields were recorded for each antibody. Granzyme B-positive cells with a sparsely granulated pattern were considered activated cytotoxic T lymphocytes.(58) Counts were performed manually with an Olympus CX31 microscope (Olympus America, Center Valley, PA, USA). First, the absolute number of lymphocytes per high-power field was determined for each antibody (CD3, CD4, CD8, Foxp3, and granzyme B; Figure2B-F). The median count number was used to divide the patients into low- and high-density groups. Second, the relative ratio of the number of cells positive for two primary antibodies was calculated, including Foxp3/CD4, granzyme B/CD8, CD4/CD8, and Foxp3/granzyme B. The median relative ratio was used to divide the patients into two groups.

4. Recurrence pattern

Recurrence diagnosed during the follow-up period was classified into four categories by modification of criteria from a previous report: loco-regional, peritoneal, systemic distant metastasis, and mixed.(59) Loco-regional recurrence included tumors in the adjacent organs, gastric bed, anastomotic site, gastric stump, or regional lymph nodes. Peritoneal recurrence included peritoneal seeding or Krukenberg's tumor. Systemic distant metastasis included extra-abdominal lymph nodes and hematogenous recurrence,

including the liver, bone, lung, or other distant sites. Mixed recurrence included those patients who met the criteria for more than one of the above categories of recurrence pattern at the time recurrence was confirmed.

5. Statistical analysis

The clinical variables evaluated were age, sex, Siewert type, Lauren classification, histologic grade, depth of invasion, nodal status, and stage. Categorical data were compared using chi-square or Fisher exact tests. Absolute numbers of cells positive for each stain, and the relative ratio between two different stains were dichotomized in the survival analysis using cut-off values derived by the median.(19, 21, 54) Overall survival and relapse-free survival curves were constructed using the Kaplan-Meier method, and the log-rank test was used to evaluate the significance. Overall survival and relapse-free survival were defined as the period from the day of surgery to death and recurrence of disease, respectively. Cox proportional hazard models were used for univariate and multivariate analysis. A statistical significance level was defined as a *p*-value of 0.05 or less. All statistical analyses were performed with SAS 9.1 software (SAS Institute, Cary, NC, USA).

RESULTS

Demographic statistics for patients

The median age of the 180 patients was 60 years and ranged from 22 to 83 years. One hundred twenty-six patients (70%) were male and 81 (45%) were Siewert type II. As for the Lauren classification, there were 76 diffuse types, 64 intestinal types, and 40 mixed types. One hundred seventy-two patients underwent total gastrectomy. Five patients underwent transhiatal esophagectomy and three patients underwent transthoracic esophagectomy. The 5-year overall survival and relapse-free survival rates were estimated to be 64.1% and 62.8%, respectively.

Univariate analysis of clinical variables

In univariate analysis of clinical variables, age group, sex, Siewert type, Lauren classification, and histologic grade were not associated with overall or relapse-free survival (**Table1**). Depth of invasion, nodal status, and stage were associated with overall and relapse-free survival in univariate analysis ($p < 0.001$ for all).

Quantification of subset of TILs

A total of nine immunologic parameters, including five simple and four combinations of lymphocyte subset results, were evaluated. The median number of cells positive for CD3, CD4, CD8, Foxp3, and granzyme B were 157.7, 92, 60.8, 15.7, and 24.5, respectively. The ratios of Foxp3/CD4, granzyme B/CD8, CD4/CD8, and Foxp3/granzyme B were 0.18, 0.44, 1.27, and 0.64, respectively. Using the median value, all cases were classified into low- and high-density groups for each variable.

Table 1. Univariate survival analysis of clinical characteristics and subsets of TILs in gastric cardia cancer.

Variables	OS			RFS		
	HR	95% CI	p-value	HR	95% CI	p-value
Clinical variable						
Age group, years (>60 vs. ≤60)	0.746	0.449-1.239	0.257	0.701	0.428-1.148	0.158
Sex (female vs. male)	1.113	0.652-1.902	0.694	1.021	0.602-1.731	0.938
Siewert type (III vs. II)	0.972	0.587-1.609	0.912	1.020	0.625-1.664	0.938
Lauren (diffuse vs. intestinal)	1.492	0.720-3.092	0.282	1.707	0.841-3.464	0.138
Histology (undifferentiated vs. differentiated)	1.463	0.862-2.481	0.158	1.632	0.970-2.746	0.065
Depth of invasion (>proper muscle vs. ≤proper muscle)	5.587	2.237-13.957	< 0.001	6.053	2.429-15.085	< 0.001
Nodal status (positive vs. negative)	7.056	3.207-15.523	< 0.001	7.850	3.578-17.221	< 0.001
Stage (III vs. I,II)	8.096	3.983-16.454	< 0.001	9.038	4.461-18.309	< 0.001
T-cell subsets						
Simple (count number)						
CD3	0.653	0.391-1.090	0.103	0.703	0.429-1.153	0.163
CD4	0.489	0.286-0.836	0.009	0.550	0.332-0.912	0.020
CD8	0.848	0.513-1.404	0.523	0.996	0.989-1.003	0.214
Foxp3	1.076	0.649-1.785	0.776	1.001	0.990-1.011	0.876
Granzyme B	0.828	0.500-1.369	0.461	0.994	0.983-1.006	0.319
Combination (ratio)						
Foxp3/CD4	2.594	1.476-4.560	0.001	2.589	1.509-4.440	0.001
Granzyme B/CD8	1.009	0.610-1.667	0.974	1.004	0.617-1.633	0.989
CD4/CD8	0.524	0.308-0.889	0.017	0.491	0.293-0.821	0.007
Foxp3/granzyme B	1.302	0.784-2.163	0.308	1.000	0.990-1.011	0.981

Identification of relevant prognostic factor in subset of TILs

In univariate analysis of subset of TILs, lower CD4, higher Foxp3/CD4 and lower CD4/CD8 was associated with poor survival and increased relapse. (Table 1, p = 0.009, p=0.001 and p=0.017 for overall survival respectively, p = 0.020, p=0.001 and p=0.007 for relapse-free survival respectively). These three immunologic parameters underwent multivariate analysis because they shared CD4 count as a common element (**Table 2**). Foxp3/CD4 was

identified as an independent prognostic factor for overall and relapse-free survival among these immunologic variables (**Figure 3**).

Table 2. Identification of relevant prognostic factors in subsets of TILs using multivariate analysis

T-cell subsets	OS*			RFS*		
	HR	95% CI	P-value	HR	95% CI	P-value
CD4	0.607	0.338-1.090	0.095	0.708	0.406-1.236	0.225
CD4 / CD3	0.930	0.503-1.716	0.815	0.867	0.476-1.582	0.642
Foxp3 / CD4	2.224	1.206-4.103	0.011	2.253	1.243-4.085	0.007

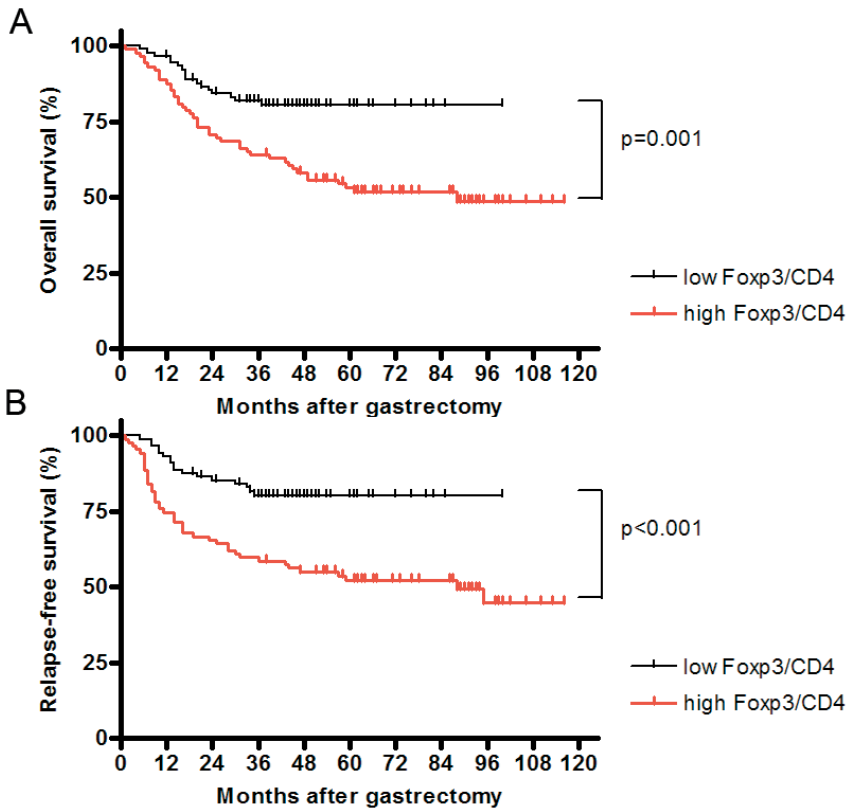

Figure 3. Kaplan-Meier analysis of overall survival and relapse-free survival for high and low Foxp3/CD4 ratio groups

Overall survival (A) and relapse-free survival (B) were plotted against the Foxp3/CD4 ratio.

Table 3. Correlation analyses between Foxp3/CD4 ratio and clinical variables

Clinical variables	Foxp3/CD4 ratio*		
	Low (n=90)	High (n=90)	p-value
Age group			0.766
Young (≤60 years)	44 (48.9%)	46 (51.1%)	
Old(>60 years)	46 (51.1%)	44 (48.9%)	
Sex			0.104
Male	68 (75.6%)	58 (64.4%)	
Female	22 (24.4%)	32 (35.6%)	
Siewert type			0.178
II	45 (50.0%)	36 (40.0%)	
III	45 (50.0%)	54 (60.0%)	
Lauren			0.474
Diffuse	34 (37.7%)	42 (46.6%)	
Intestinal	34 (37.7%)	30 (33.3%)	
Mixed	22 (24.4%)	18 (20.0%)	
Histologic grade†			0.650
Differentiated	36 (40.0%)	39 (43.3%)	
Undifferentiated	54 (60.0%)	51 (56.7%)	
Depth of invasion			< 0.001
≤proper muscle	51 (56.7%)	23 (25.6%)	
>proper muscle	39 (43.3%)	67 (74.4%)	
Nodal status			0.048
Negative	43 (47.8%)	30 (33.3%)	
Positive	47 (52.2%)	60 (66.7%)	
Stage‡			0.005
Early (I/II)	55 (61.1%)	36 (40.0%)	
Advanced (III)	35 (38.9%)	54 (60.0%)	
Recurrence site§			0.033¶
Loco-regional	0 (0.0%)	13 (28.3%)	
Peritoneal	8 (42.1%)	13 (28.3%)	
Systemic	6 (31.6%)	8 (17.4%)	
Mixed	5 (26.3%)	12 (26.1%)	
Recurrence time			1.000
Within 5 years	19 (100.0%)	44 (95.7%)	
After 5 years	0 (0.0%)	2 (4.3%)	

*Cut-off value of median, 0.18; †, Differentiated: well differentiated, moderately differentiated adenocarcinoma, undifferentiated: poorly differentiated, mucinous, signet ring cell carcinoma; ‡, AJCC 7th edition; §, loco-regional: recurrence in adjacent organs, gastric bed, anastomotic site, gastric stump or regional lymph nodes. Peritoneal recurrence: peritoneal seeding or Krukenberg's tumor. Systemic: extra-abdominal lymph nodes and hematogenous recurrence including liver, bone, lung or other distant sites; ¶, Fisher exact test.

Association of Foxp3/CD4 ratio with clinical variables and recurrence

Clinical variables, including age group, sex, Siewert type, Lauren classification, histologic grade, and recurrence time, were not associated with Foxp3/CD4 ratio (**Table 3**). More advanced depth of invasion, positive nodal status, advanced tumor stage, and local recurrence patterns were associated with a high Foxp3/CD4 ratio ($p < 0.001$, $p = 0.048$, $p = 0.005$ and $p=0.033$, respectively). In subgroup analysis for depth of invasion status, the high Foxp3/CD4 group showed a decreased survival rate without significance. In subgroup analysis for nodal status, the high Foxp3/CD4 group showed a significantly decreased survival rate only in the node-positive group ($p = 0.009$). In subgroup analysis for stage, no survival difference was observed even though the high Foxp3/CD4 group showed decreased survival ($p = 0.05$ for stages 1 and 2, $p = 0.090$ for stages 3).

Multivariate analysis for survival

Depth of invasion, nodal status, and Foxp3/CD4 ratio were used to identify independent prognostic factors of overall and relapse-free survival of the patients (**Table 4**). Multivariate analysis revealed nodal status, depth of invasion, and Foxp3/CD4 ratio as independent prognostic factors ($p = 0.002$, 0.006, and 0.042 in overall survival, $p = 0.001$, 0.003, and 0.030 in relapse-free survival, respectively).

Table 4 Multivariate analysis of clinical variables and Foxp3/CD4 ratio for overall survival and relapse-free survival

	OS			RFS		
Variables	HR	95% CI	p-value	HR	95% CI	p-value
Nodal status (positive vs. negative)	3.863	1.664-8.966	0.002	4.275	1.856-9.849	0.001
Depth of invasion (>proper muscle vs. ≤proper muscle)	3.607	1.443-9.019	0.006	3.877	1.563-9.615	0.003
Foxp3/CD4 ratio† (high vs. low)	1.812	1.022-3.212	0.042	1.837	1.062-3.177	0.030

Foxp3 = forkhead/winged helix transcription factor; †, high and low groups were classified by the median value (0.18) of the Foxp3+ cell number divided by the CD4+ cell number.

DISCUSSION

This study indicates that the distribution of intra-tumoral regulatory T cells and helper T cells, evaluated by Foxp3/CD4 ratio, is a prognostic factor of gastric cardia cancer after curative resection. Of all immunologic parameters studied, including CD3, CD4, CD8, Foxp3, granzyme B, Foxp3/CD4, granzyme B/CD8, CD4/CD8, and Foxp3/granzyme B, the Foxp3/CD4 ratio was identified as the most relevant immunological prognostic factor. The Foxp3/CD4 ratio was not associated with age group, sex, Siewert type, Lauren classification, histologic grade, or recurrence time, but was associated with depth of invasion, nodal status, and stage. Patients with more advanced status had higher Foxp3/CD4 ratios. Moreover, the high Foxp3/CD4 ratio group was associated with increased loco-regional recurrence. With ninety patients in each group, the low Foxp3/CD4 ratio group showed no loco-regional recurrence, compared with 13 patients in the high Foxp3/CD4 group.

Studies of regulatory T cells in gastric cancer are scarce, and those that are available report conflicting results. Haas et al. reported that stromal, but not intraepithelial regulatory T cells, are associated with a favorable prognosis.(21) Mizukami et al. reported that the localization pattern, but not the absolute number of regulatory T cells, was associated with prognosis.(26) On the other hand, the significance of regulatory T cells in gastric cancer as a poor prognostic factor has also been observed. Perrone et al. and Shen et al. reported unfavorable prognosis with increased intra-tumoral regulatory T cells, which is consistent with the current study.(19, 60) Previous studies used the absolute number of regulatory T lymphocytes as a parameter. However, the balance between the regulatory T lymphocytes and helper T lymphocytes is a more reliable marker of anti-tumor immune status, as it can be a

parameter of tumor-infiltrating cells skewed to favor regulatory T cells over helper T cells.(53) It has been reported that tumor-specific CD4 T cells change their phenotype from effectors to suppressors during cancer progress.(61) Conversion from effector to regulator coincided with a reduction in antigen expression levels and the induction of T-cell tolerance. Thus, the tumor microenvironment may alter the repertoire of functional tumor-specific T lymphocytes to support tumor progression.

This study showed an association between the infiltration and recurrence patterns of regulatory T lymphocytes. To my knowledge, this is the first study to investigate this association. Loco-regional recurrence might be related to the nature of regulatory T cells exerting their function in direct contact fashion. However, the mechanism of this loco-regional recurrence pattern should be investigated in future studies. In addition to recurrence pattern, regulatory T lymphocytes could be associated with late recurrence. In this study, two patients in high Foxp3/CD4 group experienced recurrence at 5 years after the surgery, although it was statistically not significant because of the small study size. It was previously reported that a high density of regulatory T-cells can identify patients with breast cancer who are at risk for relapse after 5 years.(54) Although the mechanism of late relapse is poorly understood, immunological factors are suspected to be the most relevant mechanism, and regulatory T lymphocytes might play a critical role in this process.

The limitations of this study include its retrospective observational design and the lack of several other relevant parameters, including inflammatory cytokines and regulatory T-cell infiltration in regional and distant metastatic

lymph nodes and stromal tissue. In addition, this study did not control for *H. pylori* infection status, and patients with Siewert type I cancer were not included in the study. More extensive studies including these factors should be designed and conducted in the future.

Adjuvant chemotherapy after surgery is currently decided based on the TNM staging system. The results of this study suggest that information on tumor-infiltrating lymphocytes could be used as additional piece of information on which to decide whether to begin adjuvant chemotherapy in patients with early stage disease, but with high regulatory T lymphocyte counts and to skip adjuvant chemotherapy in patients with medical comorbidity and advanced stage, but with low regulatory T lymphocyte counts. In addition, although the exact functions of each subset of tumor infiltrating lymphocytes have yet to be clarified, identification and modulation of each subset could be a promising strategy for curing gastric cancer.(62, 63) Considering the significance of regulatory T cells, as identified in this study, depletion of regulatory T cells or reversing the immunosuppressive effects of regulatory T cells may be a potent therapeutic strategy in gastric cardia cancer. In conclusion, the balance of intra-tumoral regulatory T lymphocytes and helper T lymphocytes may be a critical determinant in the prognosis of cancer patients. This study evaluated correlations among variable tumor-infiltrating T-lymphocyte subsets and prognosis in cardia cancer after curative resection. Regulatory T cells, evaluated by Foxp3/CD4 ratio, were found to be associated with an unfavorable prognosis and loco-regional recurrence.

CHAPTER 2

Systemic immune responses in gastric cancer

INTRODUCTION

Successful treatment of gastric cancer largely depends on a successful gastrectomy. While this surgery can potentially cure the disease, it also harbors the risk of perioperative morbidity and mortality. Perioperative complication rates during gastric cancer surgery range from 10% to 46%,(64-67) and adversely affect long-term survival.(68, 69) As gastric cancer is the fifth most common malignancy worldwide,(1) improving short- and long-term surgical outcomes for patients with gastric cancer is of great necessity.

Researchers have spent great effort to identify factors related with adverse surgical outcomes and prognosis. Several factors, including medical comorbidity, old age, combined resection, and advanced stage, are associated with surgical outcomes and hold prognostic significance(70-72); however, these factors are primarily unamenable, as they are related to the patient's physical or disease status. Thus, assessments of nutritional status have emerged as potential prognostic factors, since nutritional status can be corrected prior to surgery. While several tools for assessing nutritional status have been evaluated, including the nutritional risk index,(73) the nutritional risk screening 2002,(74) and subjective global assessment,(75, 76) these are difficult to use in daily clinical practice due to their complexity. Moreover, some of the parameters used by these tools are not always available, for example changes in weight.

Unlike other assessments, the prognostic nutritional index (PNI) can be easily calculated using the following equation: [(10 × serum albumin (g/dL)) + (0.005 × total lymphocyte count)].(77) The parameters used by this index are

routinely evaluated in laboratory tests during preoperative diagnostic workup and are easy-to-repeat. The predictive value of the PNI for surgical outcomes is widely accepted in various solid organ cancers, including esophageal, colorectal, liver, and pancreatic cancer.(78-81) However, only a few reports have evaluated the significance of PNI in predicting short- and long-term surgical outcomes for patients with gastric cancer,(30, 32) and a comprehensive study has never been conducted. Furthermore, controversy exists regarding the optimal cut-off values for PNI in predicting short- and long-term surgical outcomes.

Accordingly, the aim of this study was to assess the value of PNI as a predictor of perioperative morbidity and mortality, as well as a prognostic factor for recurrence-free and overall survival. Additionally, the performance of a single cut-off value based on percentiles of PNI to statistically optimized cut-off values of PNI for individually predicting morbidity, mortality, recurrence-free survival, and overall survival were compared.

MATERIALS AND METHODS

1. Patients

In the present study, data from medical records stored in a prospectively maintained database were retrospectively reviewed and analyzed. This study included 8811 consecutive patients with histologically confirmed gastric adenocarcinoma who underwent gastrectomy at Severance Hospital between January 2001 and December 2010. One thousand thirty patients with a history of other primary cancer, neoadjuvant chemotherapy, radiotherapy, non-curative resection, or emergency surgery due to perforation, bleeding, or obstruction were excluded. The remaining 7781 patients were included for analysis. The Institutional Review Board of Severance Hospital approved this study and waived the need for written informed consent from the participants (4-2015-0085).

Clinicopathological characteristics included age, sex, preoperative body mass index (BMI), medical comorbidities, American Society of Anesthesiologists (ASA) score, tumor size, and pathological stage. Perioperative data were also noted, including the extent of resection, combined resection, and operation time. Surgical resection and extent of lymphadenectomy were performed in accordance with the Japanese guidelines for treating gastric cancer.(82) Adverse events occurring within 30 days after surgery or during hospitalization were classified as postoperative complications or mortality; the type of complication was recorded. Patient staging was adjusted according to the 7th edition of the American Joint Committee on Cancer staging system.(83) Follow-up evaluations were performed according to a fixed schedule: every 3 months for 2 years, and then every 6 months for 3 years thereafter. Follow-up evaluations comprised clinical and laboratory examinations with biannual imaging and annual

endoscopic evaluation. Patients with stage II or higher disease were recommended to receive 5-fluorouracil-based adjuvant chemotherapy.

2. Prognostic nutritional index and patient grouping

Laboratory data, including serum albumin levels and lymphocyte counts, from baseline workup conducted within two months before surgery were obtained. The PNI was calculated by the following equation: [(10 × serum albumin (g/dL)) + (0.005 × total lymphocyte count)]. First, patients were divided according to every 5th percentile of PNI into 20 groups (389 patients in each group). From the 5th to 100th percentiles, the mortality events in each group were: 3, 5, 2, 1, 0, 1, 3, 0, 0, 1, 0, 1, 0, 0, 1, 0, 2, 0, 1, and 1, respectively. The complication rates for each of these groups were: 18.0%, 17.2%, 11.1%, 10.2%, 12.3%, 12.8%, 9.8%, 12.3%, 10.0%, 7.2%, 11.1%, 9.5%, 9.9%, 9.4%, 10.7%, 13.3%, 11.2%, 10.8%, 9.3%, and 11.90%, respectively. As the 10th percentile of PNI showed the highest morbidity and mortality, it was used as a cut-off to divide patients into two groups: higher or lower than the PNI value for the 10th percentile. It was hypothesized that this value would be more practical than median or mean values and could better identify patients at high risk for perioperative morbidity, as well as those who may benefit from nutritional interventions prior to surgery.

3. Statistical analysis

Categorical variables were compared using the chi-square test, and continuous variables were compared using Student's t-test. Youden's indices were used to determine the optimal PNI cut-off values to maximize sensitivity and specificity for complications and mortality.(84) Comparison

of the area under ROC curves (AUC) was performed as recommended by DeLong et al.(85)

Overall survival was defined as the duration of time from the date of surgery until the date of patient death. Recurrence-free survival was defined as the duration of time from the date of surgery until the date of histologic or radiologic recurrence of gastric cancer. To find the optimal cut-off PNI values for overall and recurrence-free survival, the Contal and O'Quigley method was used, which is based on the concept of maximizing the log-rank statistic.(86) Then the integrated areas under the curve (iAUCs) was compared between the model divided according to the 10th percentile and the models divided according to the optimized cut-off values determined using the Contal and O'Quigley method. iAUC is a weighted average of the AUC across a follow-up period and is a measure of the predictive accuracy of a model during follow-up. A higher iAUC indicates a better predictive accuracy. Differences in iAUC were calculated using a bootstrapping method with 1000 resampling times.(87)

All p-values less than 0.05 were regarded as significant, and all statistical tests were two-sided. Analyses were conducted using SAS software (version 9.2; SAS Institute, Cary, NC) and R software (version 2.13.1; R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Patient demographics and comparison of the low and high PNI groups

Table 1 lists the clinical, laboratory, operative, and pathologic characteristics of the entire cohort, and compares the characteristics for the low versus the high PNI groups. Among the entire cohort, 3624 were older than 60 years (46.6%); 5150 were male (66.2%); the mean BMI was 23.2 ± 3; and 3366 had a medical comorbidity (43.3%). Subtotal and total gastrectomies were performed in 5895 (75.8%) and 1886 (24.2%) patients, respectively. Combined resection was performed in 280 patients (3.6%). Stage I, II, and III disease was found in 4608 (59.2%), 1286 (16.5%), and 1887 (24.3%) patients, respectively. The mean PNI was 54.2 ± 5.9.

Grouping patients according to the PNI value of 46.70, it was found that low PNI was associated with old age, low BMI, medical comorbidity, a higher ASA score, low lymphocyte counts, and low albumin levels. The mean age of the patients with low and high PNI was 63.2 ± 11.1 and 56.4 ± 11.8, respectively. Operative parameters showed more frequent association between patients with a low PNI and total gastrectomy or combined resection than those with a high PNI. Patients with low PNI also had larger tumors, more advanced T and N classifications, and more advanced disease stage.

Table 1. Demographics of patients according to PNI group

		Whole Cohort, n (%); total=7781	Low PNI, n (%); total=779	High PNI, n (%); total=7002	p-value
Age group	<60	4,157 (53.4%)	245 (31.5%)	3,912 (55.9%)	<0.001
	≥60	3,624 (46.6%)	534 (68.5%)	3,090 (44.1%)	
Age		57.1 ± 11.9	63.2 ± 11.1	56.4 ± 11.8	<0.001
Sex	Male	5,150 (66.2%)	522 (67.0%)	4,628 (66.1%)	0.609
	Female	2,631 (33.8%)	257 (33.0%)	2,374 (33.9%)	
Body mass index*		23.2 ± 3	22.1 ± 2.9	23.4 ± 2.9	<0.001
Medical comorbidity	No	4,415 (56.7%)	397 (50.9%)	4,018 (57.4%)	0.001
	Yes	3,366 (43.3%)	382 (49.1%)	2,984 (42.6%)	
ASA score	1	4,327 (56.7%)	323 (42.4%)	4,004 (58.3%)	<0.001
	2	3,078 (40.4%)	382 (50.1%)	2,696 (39.3%)	
	3	218 (2.9%)	55 (7.2%)	163 (2.4%)	
	4	3 (0%)	2 (0.3%)	1 (0%)	
Lymphocytes*		2125.1 ± 663.8	1453.9 ± 454.2	2199.8 ± 641.1	<0.001
Albumin*		4.4 ± 0.4	3.6 ± 0.4	4.5 ± 0.3	<0.001
PNI value*		54.2 ± 5.9	42.8 ± 3.8	55.5 ± 4.5	<0.001
Extent of gastric resection	Subtotal gastrectomy	5,895 (75.8%)	521 (66.9%)	5,374 (76.8%)	<0.001
	Total gastrectomy	1,886 (24.2%)	258 (33.1%)	1,628 (23.2%)	
Combined resection**	No	7,501 (96.4%)	714 (91.7%)	6,787 (96.9%)	<0.001
	Yes	280 (3.6%)	65 (8.3%)	215 (3.1%)	
Operation time*		164.3 ± 53.2	166.1 ± 51.1	164.1 ± 53.4	0.317
Tumor size*		37.2 ± 26.6	58.2 ± 33.6	34.8 ± 24.6	<0.001
T classification	T1	4,182 (53.8%)	198 (25.4%)	3,984 (56.9%)	<0.001
	T2	944 (12.1%)	91 (11.7%)	853 (12.2%)	
	T3	913 (11.7%)	133 (17.1%)	780 (11.1%)	
	T4a	1,700 (21.9%)	347 (44.5%)	1,353 (19.3%)	
	T4b	42 (0.5%)	10 (1.3%)	32 (0.5%)	
N classification	N0	4,967 (63.8%)	300 (38.5%)	4,667 (66.7%)	<0.001
	N1	941 (12.1%)	124 (15.9%)	817 (11.7%)	
	N2	798 (10.3%)	124 (15.9%)	674 (9.6%)	
	N3	1,075 (13.8%)	231 (29.7%)	844 (12.1%)	
Stage	I	4,608 (59.2%)	233 (29.9%)	4,375 (62.5%)	<0.001
	II	1,286 (16.5%)	170 (21.8%)	1,116 (15.9%)	
	III	1,887 (24.3%)	376 (48.3%)	1,511 (21.6%)	

Abbreviations: PNI, prognostic nutritional index

*Student's t-test; results indicate mean ± standard deviation

**Simultaneous resection of the gallbladder owing to stone formation was not considered as a combined resection.

Comparison between the 10th percentile and statistically optimized cut-off values of PNI

Using AUC values, the performance of the 10th percentile PNI value versus statistically optimized PNI cut-off values was compared to assess overall complications, mortality, recurrence-free survival, and overall survival (**Table 2).** For short-term surgical outcomes, the optimal cut-off values determined using Youden's method for morbidity (PNI=51.52) and mortality (PNI=52.18) had higher AUCs than that of the 10th percentile value (PNI=46.70). However, no statistical difference was observed for the prediction of an event. Regarding long-term surgical outcomes, the optimal cut-offs determined by the Contal and O'Quigley method for recurrence-free survival (PNI=53.22) and overall survival (PNI=52.36) had higher iAUCs with statistically better predictive power (recurrence-free survival: ΔAUC=0.034, 95% CI=0.021-0.046; overall survival: ΔAUC=0.029, 95% CI=0.014-0.042) than that of the 10th percentile value.

Table 2. Performance of the 10th percentile value in comparison with statistically optimized cut-off values of PNI

Short-term surgical outcomes			Long-term surgical outcomes		
	AUC (95% CI)	*p-value		iAUC (95% CI)	**Δ AUC (Δ 95% CI)
Overall complications			Recurrence-free survival		
Below the 10th percentile (46.7)	0.530 (0.518-0.542)	0.590	Below the 10th percentile (46.7)	0.551 (0.541-0.562)	0.034 (0.021-0.046)
Youden's index (51.52)	0.534 (0.517-0.550)		Contal and O'Quigley method (53.22)	0.585 (0.571-0.599)	
Mortality			Overall survival		
Below the 10th percentile (46.7)	0.632 (0.529-0.735)	0.608	Below the 10th percentile (46.7)	0.566 (0.555-0.579)	0.029 (0.014-0.042)
Youden's index (52.18)	0.657 (0.554-0.760)		Contal and O'Quigley method (52.36)	0.596 (0.581-0.609)	

Abbreviations: AUC, area under the curve; iAUC, integrated area under the curve.
* Differences in AUC were calculated using the Delong method.(85)
** Differences in iAUC were calculated using a bootstrapping method with 1000 resampling times.(87)

Table 3. Short-term surgical outcomes according to PNI group

		Low PNI, n (%); total=779	High PNI, n (%); total=7002	p-value
Hospital stay*		12 ± 10.8	9.3 ± 7.9	<0.001
Overall complications	No	642 (82.4%)	6,251 (89.3%)	<0.001
	Yes	137 (17.6%)	751 (10.7%)	
Wound	No	737 (94.6%)	6,728 (96.1%)	0.047
	Yes	42 (5.4%)	274 (3.9%)	
Abscess	No	748 (96%)	6,833 (97.6%)	0.009
	Yes	31 (4%)	169 (2.4%)	
Intra-abdominal bleeding	No	774 (99.4%)	6,975 (99.6%)	0.246
	Yes	5 (0.6%)	27 (0.4%)	
Intra-luminal bleeding	No	771 (99%)	6,984 (99.7%)	0.003
	Yes	8 (1%)	18 (0.3%)	
Intestinal obstruction	No	758 (97.3%)	6,907 (98.6%)	0.003
	Yes	21 (2.7%)	95 (1.4%)	
Stenosis†	No	777 (99.7%)	6,987 (99.8%)	0.685
	Yes	2 (0.3%)	15 (0.2%)	
Leakage	No	765 (98.2%)	6,937 (99.1%)	0.022
	Yes	14 (1.8%)	65 (0.9%)	
Pulmonary complication	No	754 (96.8%)	6,856 (97.9%)	0.042
	Yes	25 (3.2%)	146 (2.1%)	
Urinary complication†	No	775 (99.5%)	6,983 (99.7%)	0.281
	Yes	4 (0.5%)	19 (0.3%)	
Renal complication †	No	775 (99.5%)	6,995 (99.9%)	0.019
	Yes	4 (0.5%)	7 (0.1%)	
Hepatic complication †	No	776 (99.6%)	6,998 (99.9%)	0.026
	Yes	3 (0.4%)	4 (0.1%)	
Cardiac complication	No	773 (99.2%)	6,980 (99.7%)	0.055
	Yes	6 (0.8%)	22 (0.3%)	
Endocrine complication †	No	779 (100%)	6,996 (99.9%)	0.999
	Yes	0 (0%)	6 (0.1%)	
Stasis†	No	777 (99.7%)	6,997 (99.9%)	0.150
	Yes	2 (0.3%)	5 (0.1%)	
Pancreas complication †	No	775 (99.5%)	6,969 (99.5%)	0.784
	Yes	4 (0.5%)	33 (0.5%)	
Mortality	No	771 (99%)	6,988 (99.8%)	<0.001
	Yes	8 (1%)	14 (0.2%)	

*Student's t-test; results indicate mean ± standard deviation

†Fisher's exact test

Short-term surgical outcomes

Patients in the low PNI group remained in the hospital longer than those in the high PNI group (**Table 3**). The overall complication and mortality rates for the entire cohort were 11.4% and 0.3%, respectively. Compared with the high PNI group, the low PNI group showed significantly higher complication rates (10.7% versus 17.6%, respectively; $p<0.001$) and mortality rates (0.2% versus 1%, respectively; $p<0.001$). The low PNI group had higher rates of wound infection, abscess formation, intra-luminal bleeding, intestinal obstruction, and leakage than the high PNI group. Complications associated with pulmonary, renal, hepatic, and cardiac organs also were observed frequently in the low PNI group. Logistic regression analysis revealed that low PNI (odds ratio [OR]=1.505, 95% CI=1.212-1.869, $p<0.001$), old age, male gender, high BMI, medical comorbidity, total gastrectomy, and combined resection were independent risk factors for overall complications (**Table 4**). Only low PNI (OR=4.279, 95% CI=1.760-10.404, $p=0.001$) and medical comorbidity were independent risk factors for mortality.

Table 4. Univariate and multivariate analyses of short- and long-term surgical outcomes

	Overall complications						Recurrence-free survival					
	Univariate			Multivariate			Univariate			Multivariate		
	OR	95% CI	p -value	OR	95% CI	p -value	HR	95% CI	p-value	HR	95% CI	p-value
Age (≥60/<60)	1.637	1.422-1.886	<0.001	1.402	1.206-1.630	<0.001	1.155	1.035-1.289	0.010	1.105	0.987-1.237	0.083
Sex (male/female)	1.355	1.161-1.582	<0.001	1.288	1.099-1.510	0.002	1.027	0.915-1.154	0.649			
Body mass index	1.049	1.025-1.074	<0.001	1.053	1.028-1.080	<0.001	0.936	0.918-0.954	<0.001	0.98	0.962-0.999	0.040
Medical comorbidity (yes/no)	1.693	1.471-1.949	<0.001	1.516	1.304-1.762	<0.001	0.927	0.829-1.037	0.183			
PNI (low/high)	1.776	1.444-2.168	<0.001	1.505	1.212-1.869	<0.001	2.430	2.110-2.798	<0.001	1.142	0.985-1.325	0.078
Gastrectomy (total/subtotal)	2.320	2.005-2.684	<0.001	2.042	1.735-2.403	<0.001	2.540	2.272-2.840	<0.001	1.194	1.056-1.351	0.005
Combined resection (yes/no)	3.190	2.432-4.184	<0.001	1.923	1.422-2.601	<0.001	5.168	4.354-6.133	<0.001	1.592	1.319-1.921	<0.001
Tumor size (≥30mm/<30)	1.427	1.241-1.642	<0.001	1.113	0.933-1.328	0.234	5.745	4.998-6.603	<0.001	1.357	1.157-1.592	<0.001
Stage			<0.001			0.827			<0.001			<0.001
II / I	1.191	0.979-1.448	0.080	0.979	0.79-1.214	0.654	6.620	5.33-8.224	<0.001	5.846	4.65-7.351	<0.001
III / I	1.558	1.328-1.828	<0.001	1.048	0.852-1.289	0.548	25.404	21.134-30.536	<0.001	19.098	15.452-23.604	<0.001
	Mortality						Overall survival					
	Univariate			Multivariate			Univariate			Multivariate		
	OR	95% CI	p -value	OR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
Age (≥60/<60)	3.068	1.199-7.849	0.019	1.562	0.594-4.106	0.366	1.821	1.649-2.011	<0.001	1.678	1.512-1.863	<0.001
Sex (male/female)	1.739	0.641-4.720	0.277				1.174	1.056-1.305	0.003	1.091	0.980-1.215	0.112
Body mass index	1.006	0.870-1.163	0.937				0.926	0.911-0.942	<0.001	0.959	0.943-0.976	<0.001
Medical comorbidity (yes/no)	13.189	3.081-56.465	0.001	11.220	2.578-48.834	0.001	1.212	1.099-1.337	<0.001	1.197	1.080-1.327	0.001
PNI (low/high)	5.179	2.166-12.385	<0.001	4.279	1.760-10.404	0.001	2.864	2.544-3.223	<0.001	1.383	1.221-1.568	<0.001
Gastrectomy (total/subtotal)	2.170	0.926-5.083	0.075				2.439	2.207-2.694	<0.001	1.346	1.205-1.502	<0.001
Combined resection (yes/no)	2.691	0.626-11.57	0.183				4.565	3.899-5.343	<0.001	1.529	1.287-1.818	<0.001
Tumor size (≥30mm/<30)	0.986	0.425-2.285	0.973				4.107	3.668-4.600	<0.001	1.337	1.166-1.534	<0.001
Stage			0.185						<0.001			<0.001
II / I	0.651	0.144-2.941	0.577				3.090	2.634-3.625	<0.001	2.605	2.198-3.087	<0.001
III / I	2.003	0.829-4.841	0.123				10.389	9.183-11.753	<0.001	7.086	6.091-8.244	<0.001

Abbreviations: OR, odds ratio; HR, hazard ratio; PNI, prognostic nutritional index.

Long-term surgical outcomes

In the recurrence-free survival analysis, patients in the low PNI group had a poor prognosis (**Figure 1 A**; p<0.001). However, after stratifying patients according to disease stage, no significant differences were found between the low and high PNI groups in recurrence-free survival for patients with stage I or stage II disease (**Figure 1 B-D;** stage I p=0.098, II p=0.076, III p=0.020). Further stratifying stage III into stages IIIa, IIIb, and IIIc also revealed no significant survival differences between the low and high PNI groups (p=0.606, p=0.461, and p=0.533, respectively). Applying the optimal PNI value determined by the Contal and O'Quigley method, recurrence-free survival still showed no survival difference between low and high PNI groups stratified by disease stage, with the exception of stage Ia (Ia p=0.008, Ib p=0.641, IIa p=0.251, IIb p=0.116, IIIa p=0.536, IIIb p=0.099, and IIIc p=0.677). Regardless of the cut-off value applied, PNI was not associated with recurrence-free survival. Using Cox regression analysis, it was found that low BMI, total gastrectomy, combined resection, larger tumor size, and stage of disease were independent risk factors of recurrence-free survival (**Table 4**). Low PNI was not an independent risk factor for recurrence-free survival (hazard ratio [HR]=1.142, 95% CI=0.985-1.325, p=0.078).

In the overall survival analysis, the low PNI group had a poor prognosis for all stages of disease (**Figure 1 E-H**; for all stages and stage I, II, and III: p<0.001). Independent risk factors for overall survival included low PNI (HR=1.383, 95% CI=1.221-1.568, p<0.001), old age, low BMI, medical comorbidity, total gastrectomy, combined resection, larger tumor size, and disease stage.

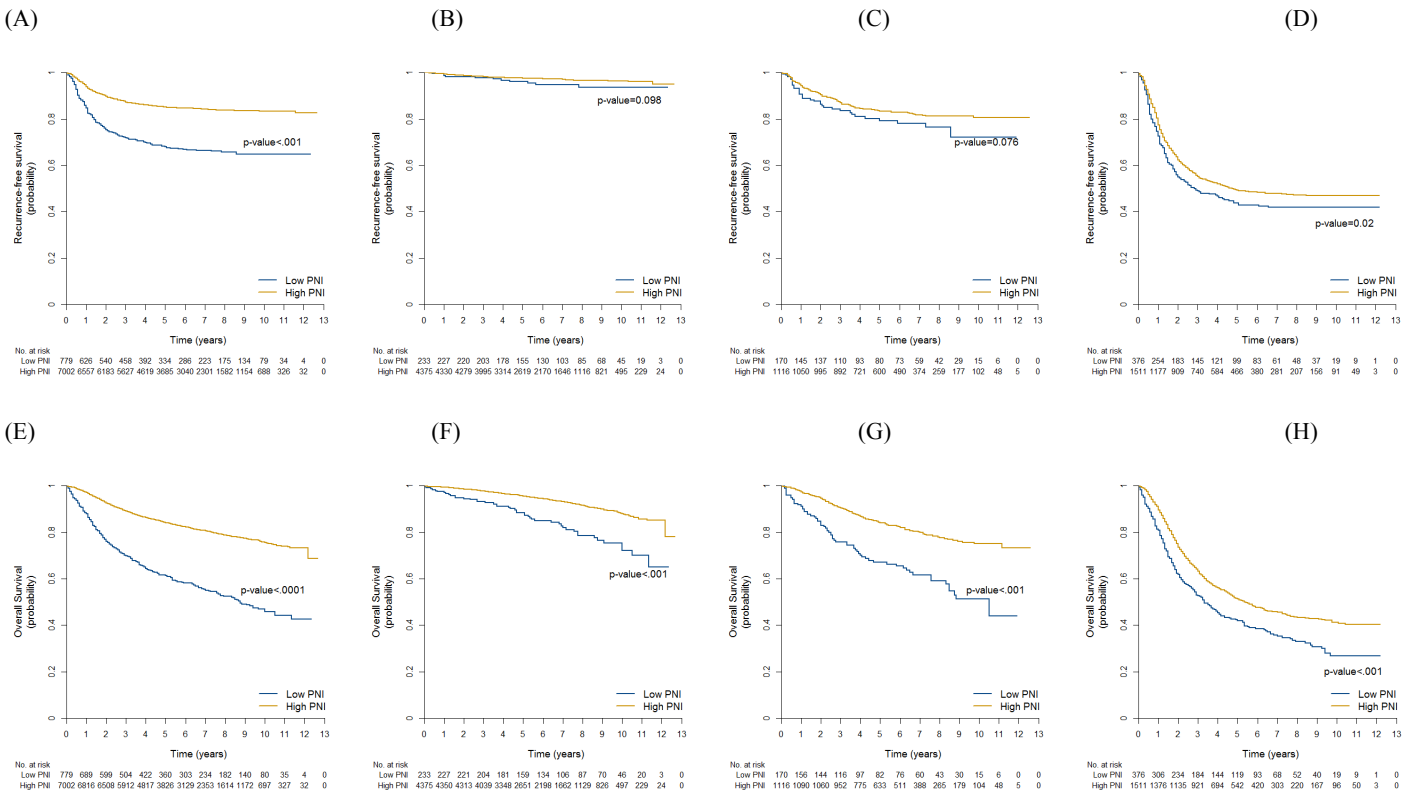

Figure 1. Representative cases of TILs in cardia and non-cardia tumor samples

Recurrence-free survival of (A) all stages, (B) stage I, (C) stage II, and (D) stage III. Overall survival of (E) all stages, (F) stage I, (G) stage II, and (H) stage III. PNI, prognostic nutritional index; HR, hazard ratio.

Comparison after adjustment for confounding factors

To account for confounding factors in evaluating the performance of each cut-off value, a stepwise adjustment for confounding factors to develop models for short- and long-term surgical outcomes was applied (**Table 5**). Both the 10th percentile cut-off value and the statistically optimized cut-off values showed robustness after adjusting for confounding variables. Interestingly, the 10th percentile value showed higher odds ratios and hazard ratios with more statistical significance than the statistically optimized cut-off values for mortality, recurrence-free survival and overall survival.

Table 5. Performance of the cut-off values after adjustment for confounding factors

Short-term surgical outcomes							Long-term surgical outcomes								
Adjustment	Model 1		Model 2		Model 3		Adjustment	Model 1		Model 2		Model 3		Model 4	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value		HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Overall complications							Recurrence-free survival								
Below the 10th percentile (46.7)	1.776 (1.455-2.168)	<0.001	1.686 (1.369-2.077)	<0.001	1.501 (1.214-1.856)	0.002	Below the 10th percentile (46.7)	2.43 (2.11-2.798)	<0.001	2.295 (1.987-2.651)	<0.001	2.014 (1.742-2.33)	<0.001	1.167 (1.009-1.351)	0.038
Youden's index (51.52)	1.380 (1.190-1.6)	<0.001	1.321 (1.131-1.544)	0.005	1.192 (1.017-1.397)	<0.001	Contal and O'Quigley method (53.22)	2.039 (1.826-2.276)	<0.001	1.966 (1.757-2.2)	<0.001	1.774 (1.584-1.987)	<0.001	1.081 (0.963-1.213)	0.185
Mortality							Overall survival								
Below the 10th percentile (46.7)	5.179 (2.166-12.385)	<0.001	4.631 (1.931-11.107)	<0.001			Below the 10th percentile (46.7)	2.864 (2.544-3.223)	<0.001	2.274 (2.009-2.575)	<0.001	2.073 (.832-2.346)	<0.001	1.337 (1.179-1.516)	<.001
Youden's index (52.18)	3.681 (1.542-8.787)	0.003	3.525 (1.475-8.425)	0.004			Contal and O'Quigley method (52.36)	2.271 (2.06-2.504)	<0.001	1.908 (1.722-2.113)	<0.001	1.751 (1.58-1.94)	<0.001	1.164 (1.048-1.293)	0.004

Abbreviations: CI, confidence interval; HR, hazard ratio; OR, odds ratio.

Adjusted variables for overall complications:

Model 1=PNI; Model 2=PNI, age, sex, BMI, medical comorbidity; Model 3=PNI, age, sex, BMI, medical comorbidity, gastrectomy, combined resection

Adjusted variables for mortality:

Model 1=PNI; Model 2=PNI, medical comorbidity

Adjusted variables for recurrence-free survival:

Model 1=PNI; Model 2=PNI, BMI; Model 3=PNI, BMI, gastrectomy, combined resection; Model 4=PNI, BMI, gastrectomy, combined resection, tumor size, stage

Adjusted variables for overall survival:

Model 1=PNI; Model 2=PNI, age, BMI, medical comorbidity; Model 3=PNI, age, BMI, medical comorbidity, gastrectomy, combined resection; Model 4=PNI, age, BMI, medical comorbidity, gastrectomy, combined resection, tumor size, stage

DISCUSSION

The present study retrospectively analyzed individual clinical data from 7781 patients who underwent curative resection for gastric cancer at a high-volume center in Korea. Selecting the 10th percentile PNI value as a cut-off, it was found that patients with a PNI lower than 46.70 show significantly higher overall morbidity and mortality than those with a higher PNI. Low PNI also was associated with unfavorable overall survival; recurrence-free survival was not correlated with PNI.

In the literature, various cut-off values for PNI have been suggested, including 49.7,(88) 48,(30) and 44.7.(32) This study used the 10th percentile PNI value (46.70) and statistically optimized values as cut-offs for overall complications (51.52, the 28.4th percentile), mortality (52.18, the 32.3rd percentile), recurrence-free survival (53.22, the 39.6th percentile), and overall survival (52.36, the 33.4th percentile). Clinically, a cut-off value higher than the 10th percentile value, including median or mean values, would not be useful, as too many patients would be categorized as high risk. In this study, patients with a PNI value in the 10th percentile showed a mortality rate five times higher than that of other patients. The strength of this study is that it could validate the use of PNI to predict mortality, which is an extremely rare event, in a very large cohort. Additionally, it successfully demonstrated the robustness of the 10th percentile PNI value in comparison to optimal cut-off values for individual short- and long-term surgical outcomes.

Numerous prospective studies of perioperative nutritional support have failed to reveal improvements in short-term surgical outcomes as a result thereof.(89-91) Thus, it is likely that only severely malnourished patients benefit from preoperative nutritional support.(91-93) If malnutrition affects postoperative results and a clinically applicable parameter becomes available, interventions to improve

nutritional status prior to surgery could become attractive targets to optimize patient outcomes. Since it is unknown whether PNI could serve as a nutritional parameter to select candidates for nutritional intervention, prospective validation of nutritional intervention in patients with low PNI should be performed in the future.

Regarding long-term surgical outcomes, this study showed that PNI was an independent risk factor for overall survival, but not for recurrence-free survival. Since these findings on recurrence-free survival do not corroborate those of a previous study,(30) this study extensively validated the prognostic significance of PNI. For further validation, the performance of the 10th percentile cut-off value in comparison to optimal cut-off values derived from statistical tests were examined. In doing so, it was found that low PNI is indeed not a significant prognostic factor for recurrence-free survival in subgroup analysis stratifying patients by disease stage or in multivariate Cox analysis. Therein, the prognostic impact of PNI on recurrence-free survival decreased and disappeared after adjusting for confounding factors. Additionally, although PNI was significantly associated with overall survival, an age difference of 7 years (63.2 vs. 56.4) between the two groups may have affected the survival analysis results, despite adjusting for age in the statistical models. Contrary to previous reports, findings of current study showed PNI holds little prognostic value as a parameter for long-term surgical outcomes.

Despite extensive validation in a large cohort, retrospective inclusion and exclusion of patients, the collection of laboratory data, and the use of prospectively maintained databases, this study has inherent limitations related with its retrospective design. This study did not control for other variables affecting PNI. However, to the best of my knowledge, this study is the most comprehensive, to date and includes extensive

comparison with statistically optimized cut-off values and adjustment for potential confounding factors.

In conclusion, PNI was not associated with cancer recurrence in the present study. Although low PNI patients showed unfavorable prognosis regarding overall survival, their advanced age may have affected the survival results, despite adjusting for age in multivariate analysis. The index, nevertheless, exhibits predictive capabilities for the stratification of patients at increased risk of postoperative morbidity and mortality. Moreover, this index may be of use in identifying candidate patients who would benefit from perioperative nutritional support to improve surgical outcomes.

CHAPTER 3

Local and systemic immune responses in cardia and non-cardia cancer

INTRODUCTION

Tumor infiltrating lymphocytes (TILs) are known to be prognostic factors in various solid organ cancers. In gastric cancer, regulatory T cells, the most extensively studied subset of TILs, are thought to induce tolerance to altered self-antigens, resulting in a deleterious immune response by the host. Although poor prognostic power for regulatory T cells has been reported,(11-20) some researchers have reported a favorable prognostic impact for regulatory T cells.(21-23) However, reported risk assessments were not adjusted to include stage(15, 20) or lacked clinically relevant information, such as tumor location.(11, 16, 18, 20, 24-27)

Thus, this study analyzed the prognostic impact of immunological parameters in the context of clinicopathological parameters. Recent studies have identified distinct molecular and pathophysiologic features of cardia and non-cardia cancer.(94, 95) Cardia cancers are associated with gastroesophageal reflux(41, 42) and obesity.(41, 43-45) The incidence of cardia cancers has remained stable or has increased in recent analyses.(41, 46-48) In contrast, non-cardia cancers are known to be caused by *Helicobacter pylori* infection,(49) and the incidence of this type of cancer is actually decreasing.(42, 47-49) This study hypothesized that the location of the tumor in the gastric tube would be associated with different clinicopathological and immunological characteristics.

In addition, contrary to TILs that reflect local immune responses in the host, prognostic nutritional index (PNI) and neutrophil-to-lymphocyte ratio (NLR) can be used as parameters of systemic immune responses. The value of these parameters has been identified in various types of cancers, including gastric cancer.(28-31) Moreover, PNI and NLR can be easily calculated with information obtained during routine

preoperative laboratory examination, including albumin levels, lymphocyte counts, and neutrophil counts. However, the value of PNI and NLR has rarely been studied within the context of cancer TILs,(33-36) and has never been studied in gastric cancer patients. Therefore, the aim of the study was to characterize the prognostic impact of TILs (a marker of local immune responses) in association with PNI and NLR (markers of systemic immune responses) according to tumor location within the stomach.

MATERIALS AND METHODS

1. Study design and patients

A prospectively collected database of gastric cancer patients was retrospectively analyzed. Four-hundred sixteen patients with gastric cancer that underwent curative resection at Severance Hospital were enrolled in the study. Surgeries were performed from January 2000 to June 2011. Follow-up of the patients ended on August 25, 2015, with a median follow-up time of 89.5 months. Inclusion criteria were gastric adenocarcinoma without distant metastasis and curative surgical resection with or without adjuvant chemotherapy. Excluded patients comprised those that received neoadjuvant chemotherapy or radiation therapy, those with a history of other primary cancers, and those who died within 30 days of surgery. Patients with poor paraffin block status for immunohistochemical staining or unclear information with regards to the longitudinal location of the tumor were also excluded. Staging and histologic grade were recorded according to the American Joint Committee on Cancer 7th Edition. (83) Patients with stage II-III cancer were considered for 5-fluorouracil-based adjuvant chemotherapy according to the standard treatment regimen. Patients underwent follow-up examinations every three months for one year, every six months for two years, and every year thereafter for the duration of the scheduled follow-up period. This study was approved by the Yonsei Institutional Review Board (4-2017-0753).

2. Local immune responses

Immunohistochemical staining and quantification of TILs were performed as previously described. (17) Briefly, immunohistochemical staining was performed on paraffin-embedded cancer tissue sections that had been serially sectioned at 4-mm thickness after using hematoxylin and eosin staining. The sections were deparaffinized, rehydrated, and treated for antigen retrieval. The sections were

incubated for 60 min at room temperature with primary monoclonal antibodies (**Figure 1**): CD3 (1:100, Labvision Corporation, Fremont, CA), CD4 (1:100, Novocastra, Newcastleupon Tyne, UK), CD8 (1:100, Novocastra), Foxp3 (1:100, Abcam, Cambridge, UK), and granzyme B (1:100, Labvision Corporation). These antibodies were used to identify the following T lymphocyte subsets: total T lymphocytes, helper T lymphocytes, cytotoxic T lymphocytes, regulatory T cells, and activated cytotoxic T lymphocytes, respectively. Incubation in horseradish peroxidase-conjugated secondary antibody was subsequently performed, followed by development with diaminobenzidine and counterstaining with hematoxylin.

An experienced pathologist blinded to the patient data reviewed the histological slides. Five high-power fields (400x) from each slide were selected for immunohistochemical evaluation. The mean number of positively stained cells per high-power fields were recorded for each antibody. Counts were performed using an Olympus CX31 microscope (Olympus America, Center Valley, PA) and ImageJ software (http://rsb.info.nih.gov/ij). The absolute number of lymphocytes per high-power field was determined for each antibody.

3. Systemic immune responses

Calculations of PNI and NLR were performed as previously described. (29) Laboratory data, including serum albumin levels, neutrophil counts, and lymphocyte counts, from baseline workup conducted within two months before surgery were obtained. The PNI was calculated by the following equation: [(10 × serum albumin (g/dL)) + (0.005 × total lymphocyte count)]. The NLR was calculated by the following equation: (neutrophil count / lymphocyte count).

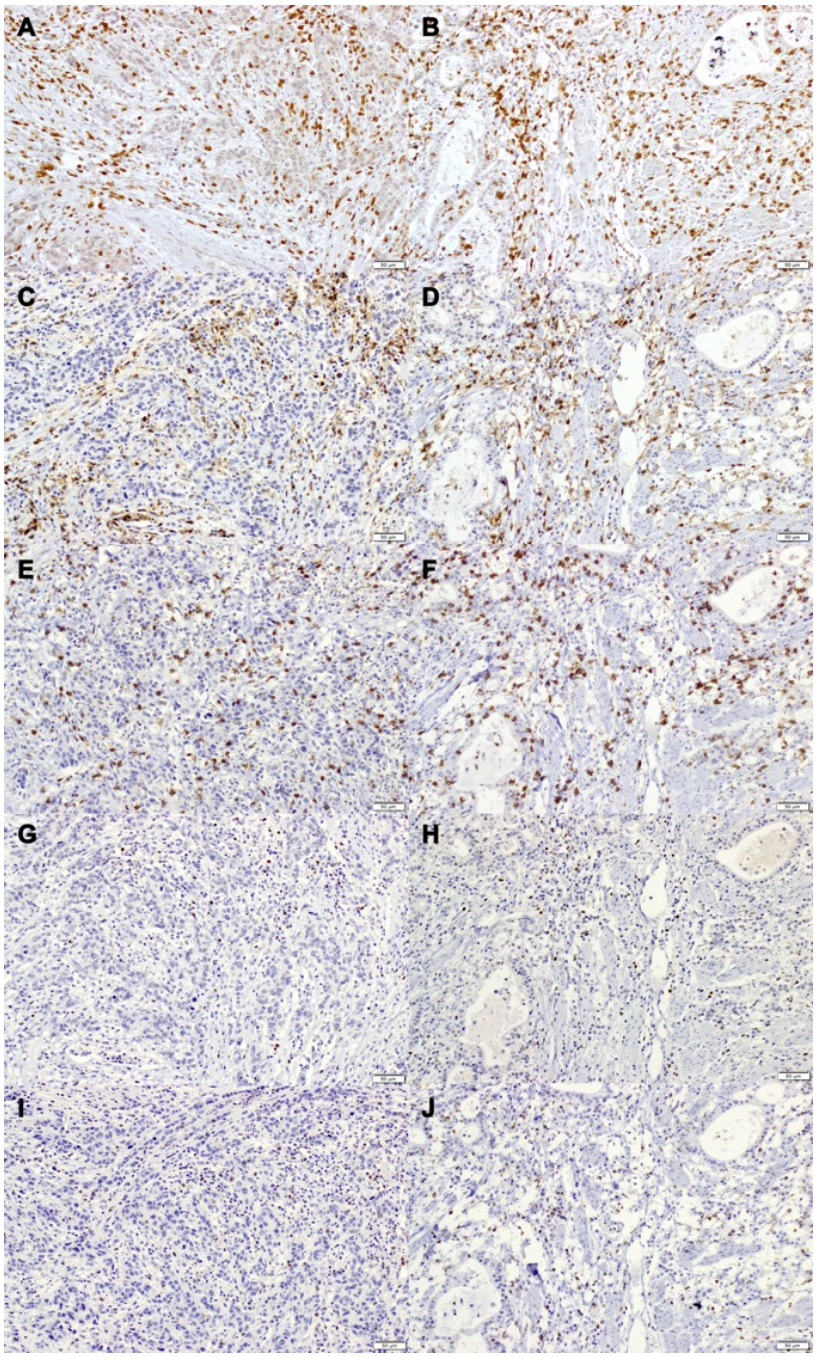

Figure 1. Representative cases of TILs in cardia and non-cardia tumor samples

Immunohistochemical staining of CD3 (**A, B**), CD4 (**C, D**), CD8 (**E, F**), Foxp3 (**G, H**), and Granzyme B (**I, J**) in cardia and non-cardia human gastric adenocarcinoma tumor samples (original magnification, x200).

4. Statistical analyses

The clinical variables evaluated were age, sex, body mass index, tumor size, circular location, longitudinal location, histologic grade, histologic type, Lauren classification, lymphovascular invasion, perineural invasion, depth of invasion, nodal status, and stage. Categorical data were compared using chi-square or Fisher exact tests, and continuous variables were compared using Student's t test or analysis of variance. Correlation between two continuous variables were evaluated using Pearson's method. Absolute numbers of cells positive for each stain and the relative ratio between two different stains were dichotomized using cutoff values derived by the median. Survival curves were constructed using the Kaplan-Meier method, and the log-rank test was used to evaluate significance. Cox proportional hazards models were used for univariate analysis. A multivariate Cox forward stepwise regression model was used to detect independent predictors of survival. The factors with *p*-values of < 0.10 on univariate analysis were entered into the multivariate analysis. A statistical significance level was defined as a *p*-value of 0.05 or less. All statistical analyses were performed with SAS 9.1 software (SAS Institute, Cary, NC).

Table 1. Association between clinical parameters and immune responses

Characteristics		Local immune response										Systemic immune response			
	Number	CD3	p-value*	CD4	p-value*	CD8	p-value*	Foxp3	p-value*	Granzyme B	p-value*	PNI	p-value*	NLR	p-value*
Age (years)			0.268		0.885		0.931		0.569		0.687		0.007		0.756
<60	201 (48.3%)	172.2 ± 67.5		96.9 ± 52.4		79.8 ± 42.0		19.4 ± 14.8		21.8 ± 19.2		53.6 ± 5.7		2.2 ± 1.1	
≥60	215 (51.7%)	165.3 ± 58.9		96.1 ± 50.0		79.5 ± 35.2		20.3 ± 14.9		21.0 ± 17.9		52.0 ± 6.4		2.3 ± 1.6	
Gender			0.251		0.661		0.118		0.854		0.487		0.988		0.108
Male	273 (65.6%)	166.0 ± 60.8		97.3 ± 52.5		81.8 ± 38.4		20.0 ± 14.9		21.8 ± 18.2		52.8 ± 6.6		2.3 ± 1.3	
Female	143 (34.4%)	173.5 ± 67.7		95.0 ± 48.5		75.6 ± 38.8		19.7 ± 14.7		20.4 ± 19.1		52.8 ± 5.0		2.1 ± 1.4	
BMI			0.807		0.549		0.941		0.666		0.432		<0.001		0.041
Low	205 (49.3%)	167.8 ± 63.7		98.3 ± 53.9		79.3 ± 40.5		20.2 ± 16.6		20.7 ± 15.7		51.7 ± 5.6		2.3 ± 1.2	
High	205 (49.3%)	169.3 ± 63.2		95.2 ± 48.8		79.6 ± 36.9		19.5 ± 13		22.2 ± 21.0		54.0 ± 6.4		2.1 ± 1.2	
Tumor> 4cm			0.711		0.273		0.216		0.412		0.050		<0.001		<0.001
No	201 (48.3%)	167.4 ± 64.8		99.3 ± 53.2		77.2 ± 36.0		19.2 ± 16.5		19.5 ± 16.8		54.6 ± 5.3		2.0 ± 1.1	
Yes	215 (51.7%)	169.7 ± 61.9		93.8 ± 49.1		81.9 ± 40.8		20.4 ± 13.1		23.1 ± 19.8		51.1 ± 6.3		2.5 ± 1.5	
Histologic type			0.683		0.517		0.777		0.265		0.407		0.038		0.114
Papillary	3 (0.7%)	122.4 ± 13.7		74.9 ± 27.1		85.0 ± 19.1		19.3 ± 10.5		12.4 ± 6.9		51.3 ± 4.8		2.0 ± 0.3	
Well-differentiated	53 (12.7%)	163.1 ± 62.9		99.0 ± 52.9		75.0 ± 32.2		17.1 ± 16.6		17.6 ± 14.1		53.6 ± 6.9		2.0 ± 1.3	
Mod-differentiated	127 (30.5%)	165.8 ± 63.4		91.8 ± 48.2		80.0 ± 35.6		22.4 ± 15.8		22.7 ± 18.3		51.8 ± 5.9		2.3 ± 1.1	
Poorly-differentiated	164 (39.4%)	173.0 ± 62.5		98.5 ± 51.6		82.5 ± 41.6		19.1 ± 13.9		22.4 ± 20.8		52.5 ± 5.9		2.4 ± 1.7	
Mucinous	13 (3.1%)	173.6 ± 56.7		117.6 ± 61.2		76.8 ± 20.1		20.7 ± 12.4		15.8 ± 11.8		56.0 ± 6.0		2.4 ± 0.9	
Signet ring cell type	56 (13.5%)	168.6 ± 68.6		95.2 ± 53.1		75.3 ± 45.1		18.8 ± 14.0		21.0 ± 16.7		54.3 ± 6.0		1.9 ± 0.7	
Lauren			0.515		0.017		0.848		0.430		0.076		0.057		0.584
Intestinal	165 (39.7%)	170.9 ± 57.9		97.3 ± 47.9		80.2 ± 36.2		20.5 ± 14.5		20.4 ± 17.7		52.6 ± 6.3		2.3 ± 1.4	
Diffuse	119 (28.6%)	177.2 ± 60.9		101.4 ± 50.4		80.5 ± 42.1		18.7 ± 13.6		18.6 ± 15.3		53.6 ± 6.1		2.1 ± 1.1	
Mixed	30 (7.2%)	181.9 ± 61.1		126.4 ± 66.4		84.6 ± 42.9		17.7 ± 11.6		27.3 ± 29.0		55.5 ± 4.7		2.2 ± 1.0	
Lymphovascular invasion			0.165		0.636		0.090		0.891		0.156		0.001		0.060
No	117 (28.1%)	166.7 ± 58.9		99.3 ± 51.2		75.6 ± 39.8		16.5 ± 9.4		16.8 ± 16.4		54.8 ± 5.8		2.1 ± 1.1	
Yes	162 (38.9%)	176.3 ± 55.2		102.3 ± 53.5		83.4 ± 35.6		16.3 ± 9.1		19.9 ± 17.9		52.4 ± 5.9		2.4 ± 1.4	
Perineural invasion			0.599		0.574		0.704		0.061		0.116		0.178		0.446
No	155 (37.3%)	171.8 ± 54.7		101.5 ± 51.9		79.2 ± 35.7		17.2 ± 9.5		16.4 ± 13.1		53.9 ± 6.2		2.2 ± 1.4	
Yes	107 (25.7%)	175.4 ± 55.3		105.2 ± 54.5		80.9 ± 38.4		15.1 ± 8.4		19.8 ± 20.4		52.9 ± 5.7		2.3 ± 1.2	
T classification			0.070		0.958		0.010		0.012		0.001		<0.001		0.011
T1, T2	162 (38.9%)	161.6 ± 61.9		96.7 ± 48.3		73.6 ± 35.0		17.6 ± 12.5		17.8 ± 13.9		54.4 ± 5.7		2.4 ± 1.4	
T3, T4	254 (61.1%)	173.1 ± 63.8		96.4 ± 52.9		83.5 ± 40.3		21.3 ± 16.0		23.8 ± 20.7		51.8 ± 6.1		2.2 ± 1.4	
N classification			0.597		0.056		0.868		0.708		0.643		0.003		0.164
N0	197 (47.4%)	166.6 ± 62.8		101.1 ± 51.9		80.7 ± 40.7		20.7 ± 16		21.4 ± 19.1		53.5 ± 6		2.2 ± 1.6	
N1	53 (12.7%)	177.2 ± 66.9		104.4 ± 55.9		79.0 ± 41.9		19.0 ± 11.8		23.1 ± 19.6		51.9 ± 7		2.3 ± 1.1	
N2	70 (16.8%)	163.5 ± 62.7		84.7 ± 43.3		76.3 ± 31.0		18.7 ± 12.2		19.0 ± 17.4		53.8 ± 5.1		2.0 ± 0.9	
N3	96 (23.1%)	171.9 ± 62.7		91.4 ± 50.7		80.3 ± 37.4		19.4 ± 15.7		22.2 ± 17.4		51.0 ± 6		2.4 ± 1.2	
Stage			0.086		0.033		0.002		0.057		0.107		<0.001		0.147
I	131 (31.5%)	159.6 ± 63.5		97.5 ± 49.8		72.1 ± 35.1		18.2 ± 13.6		18.6 ± 14.5		54.6 ± 5.6		2 ± 1.4	
II	115 (27.6%)	177.4 ± 57.9		105.6 ± 52.9		89.5 ± 44.2		22.6 ± 16.1		23.3 ± 21.9		51.7 ± 6.7		2.4 ± 1.6	
III	170 (40.9%)	169.7 ± 65.9		89.6 ± 50.2		78.8 ± 35.8		19.3 ± 14.7		22.3 ± 18.6		52.1 ± 5.7		2.3 ± 1.1	

*The comparisons between groups were made with the independent samples *t* test or analysis of variance.

RESULTS

Patient demographics with local and systemic immune responses

Table 1 shows clinicopathological parameters associated with local and systemic immune responses of patients. For local immune responses, CD3 and CD4 had no association with clinicopathological characteristics, except that CD4 was significantly associated with Lauren classification and stage ($p = 0.017$ and $p = 0.033$, respectively). High CD8 levels were associated with advanced T-classification and final stage ($p = 0.010$, and $p = 0.002$, respectively). High Foxp3 and granzyme B levels were associated with advanced T-classification ($p = 0.012$, and $p = 0.001$, respectively). For systemic immune responses, high PNI was associated with younger age, high BMI, small tumor size, mucinous type, no lymphovascular invasion, less advanced T-classification, N-classification, and final stage. High NLR was associated with low BMI, larger tumor size, and less advanced T-classification. Of note, histologic type, Lauren classification, lymphovascular invasion, and perineural invasion had little association with local and systemic immune responses.

Tumor location with local and systemic immune responses

Figure 2 shows the anatomic locations associated with local and systemic immune responses of the patients. For longitudinal location, CD3 and CD4 levels had no association with location, whereas CD8 counts were significantly different between the cardia and antrum (**Fig. 2A**). Foxp3 and granzyme B counts were also significantly higher in the cardia, compared with other locations (**Fig. 2C-D**). PNI and NLR values showed no differences among longitudinal locations (**Fig. 2E-H**). For circular location, no association was found with local or systemic immune responses (data not shown).

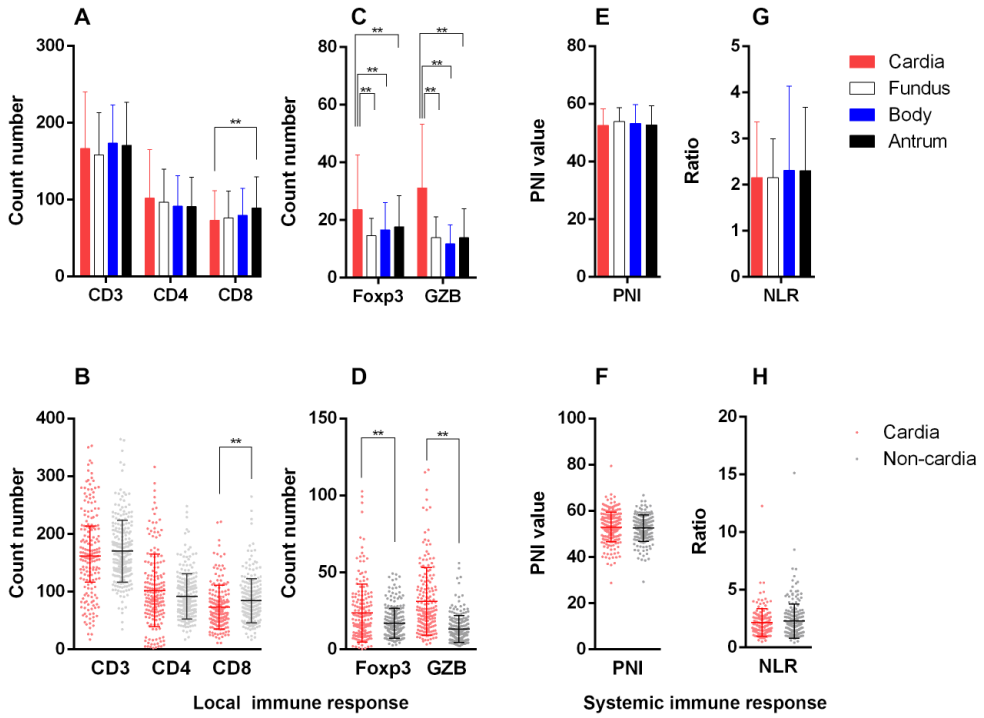

Figure 2. Relationship of local and systemic immune responses with tubular location of the tumor (A-D) for local immune responses, cardia was associated with significantly higher number of CD8, Foxp3, and granzyme B TILs. **(E-F)** for systemic immune responses, PNI showed no association. **(G-H)** NLR showed no association.

**p<0.01; Foxp3, forkhead/winged helix transcription factor; GZB, granzyme B; PNI, prognostic nutritional index; NLR, neutrophil to lymphocyte ratio

Table 2. Comparison of cardia and non-cardia

	Characteristics	Cardia n=181	Non-cardia n=235	p-value
Clinical	Age (years)			0.773
	<60	86 (47.5%)	115 (48.9%)	
	≥60	95 (52.5%)	120 (51.1%)	
	Gender			0.277
	Male	124 (68.5%)	149 (63.4%)	
	Female	57 (31.5%)	86 (36.6%)	
	BMI	23.2±3.2	23±3.2	0.657
Pathological	Tumor > 4cm			0.140
	No	80 (44.2%)	121 (51.5%)	
	Yes	101 (55.8%)	114 (48.5%)	
	Histologic type			0.265
	Papillary	0 (0%)	3 (1.3%)	
	Well-differentiated	21 (11.6%)	32 (13.6%)	
	Mod-differentiated	64 (35.4%)	63 (26.8%)	
	Poorly-differentiated	67 (37%)	97 (41.3%)	
	Mucinous	4 (2.2%)	9 (3.8%)	
	Signet ring cell type	25 (13.8%)	31 (13.2%)	
	Lauren			0.082
	Intestinal	71 (58.2%)	94 (49%)	
	Diffuse	37 (30.3%)	82 (42.7%)	
	Mixed	14 (11.5%)	16 (8.3%)	
	Lymphovascular invasion			0.072
	No	32 (34.4%)	85 (45.7%)	
	Yes	61 (65.6%)	101 (54.3%)	
	Perineural invasion			0.364
	No	44 (55%)	111 (61%)	
	Yes	36 (45%)	71 (39%)	
	T classification			0.003
	T1, T2	56 (30.9%)	106 (45.1%)	
	T3, T4	125 (69.1%)	129 (54.9%)	
	N classification			0.393
	N0	77 (42.5%)	120 (51.1%)	
	N1	25 (13.8%)	28 (11.9%)	
	N2	33 (18.2%)	37 (15.7%)	
	N3	46 (25.4%)	50 (21.3%)	
	Stage			0.031
	I	49 (27.1%)	82 (34.9%)	
	II	45 (24.9%)	70 (29.8%)	
	III	87 (48.1%)	83 (35.3%)	
Tumor location	Circular location			0.007
	Lesser curvature	92 (53.5%)	104 (44.6%)	
	Greater curvature	12 (7%)	43 (18.5%)	
	Anterior wall	29 (16.9%)	31 (13.3%)	
	Posterior wall	39 (22.7%)	55 (23.6%)	
Local immune response	CD3	166.6 ± 73.9	170.2 ± 53.7	0.586
	CD4	102.1 ± 63	92.1 ± 39.2	0.062
	CD8	73.5 ± 38.2	84.4 ± 38.3	0.004
	Foxp3	23.6 ± 18.9	17 ± 9.8	<0.001
	Granzyme B	31.2 ± 22	13.2 ± 8.8	<0.001
	Foxp3/CD4 (%)	31.4 ± 29.3	19.8 ± 11.7	<0.001
Systemic immune response	NLR	53 ± 6.4	52.5 ± 5.7	0.451
	PNI	2.3 ± 1.5	2.2 ± 1.2	0.336

Foxp3, forkhead/winged helix transcription factor; PNI, prognostic nutritional index; NLR, neutrophil to lymphocyte ratio

Comparison of cardia and non-cardia according to local and systemic immune responses

Cardia and non-cardia location showed little difference in its association with clinicopathologic parameters, except that advanced T-classification and stage were correlated with cardia cancer (**Table 2**). For local immune responses, cardia lesions showed significantly higher CD8 counts ($p = 0.004$), smaller Foxp3 counts ($p < 0.001$), smaller granzyme B counts ($p < 0.001$), and a higher Foxp3/CD4 ratio ($p < 0.001$). For systemic immune responses, neither NLR nor PNI had a significant difference in association with cardia or non-cardia lesions.

Association of local and systemic immune responses

Local immune responses were evaluated using CD3, CD4, CD8, Foxp3, and granzyme B levels. Local immune responses showed poor correlation with systemic immune response (**Figure 3**), with only marginal negative association between CD4 and NLR (**Figure 3G**, $p = 0.040$)

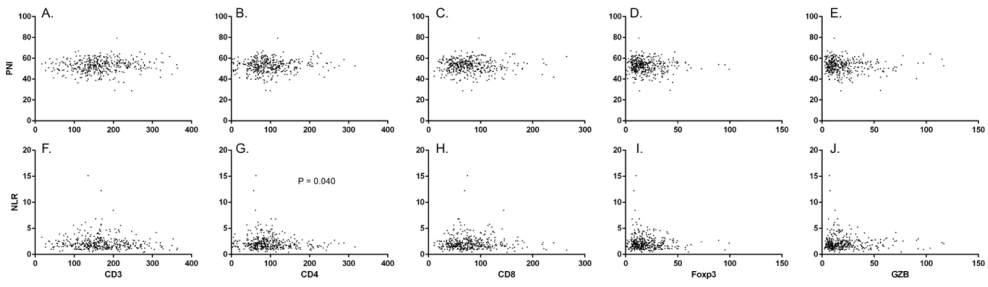

Figure 3. Association of local and systemic immune responses

First row (**A-E**) and second row (**F-J**) shows prognostic nutritional index and neutrophil-to-lymphocyte ratio, respectively. These were plotted against CD3 (**A, F**), CD4 (**B, G**), CD8 (**C, H**), Foxp3 (**D, I**), and granzyme B (**E, J**), respectively. Only CD4 and NLR showed a significant association (**G**, $p = 0.040$).

Prognostic implications of local and systemic immune responses according to tumor location

To determine if the location of a tumor and immune responses affected prognosis, survival analysis was performed, and the results are depicted in **Figure 4.** CD3, CD8 and granzyme B showed no survival difference regardless of tumor location (data not shown). High Foxp3 levels showed an unfavorable prognostic impact in cardia cancers (**Figure 4E, p = 0.024**) and a favorable prognostic impact in non-cardia cancers (**Figure 4F, p = 0.035**). Additionally, high PNI value was a good prognostic factor only in the non-cardia group (**Figure 4L, p = 0.002**), while high NLR value was a poor prognostic factor only in the cardia group (**Figure 4N, p = 0.008**).

In univariate analysis for the whole cohort, total gastrectomy, larger tumor size, advanced T-classification, node metastasis, low CD4 count, high Foxp3/CD4 ratio, and low PNI value were all poor prognostic factors (**Table 3**). Multivariate analysis revealed that T-classification (HR = 2.158, p = 0.001), node positivity (HR = 2.274, p < 0.001), and PNI (HR = 0.953, p < 0.001) were independent prognostic factors. In subgroup analysis for cardia cancer, node positivity (HR = 3.347, p < 0.001) and high Foxp3/CD4 ratio (HR = 1.008, p = 0.019) were independent unfavorable prognostic factors. In non-cardia cancer, total gastrectomy (HR = 1.596, p = 0.045), T-classification (HR = 2.640, p = 0.001), node positivity (HR = 1.781, p = 0.028), low Foxp3 count (HR = 0.968, p = 0.017), and low PNI value (HR = 0.935, p = 0.001) were independent unfavorable prognostic factors. For recurrence free survival, neither systemic nor local inflammatory response was identified as a risk factor for cardia cancer, whereas high foxp3/CD$ and PNI were good prognostic factor for non-cardia cancer.

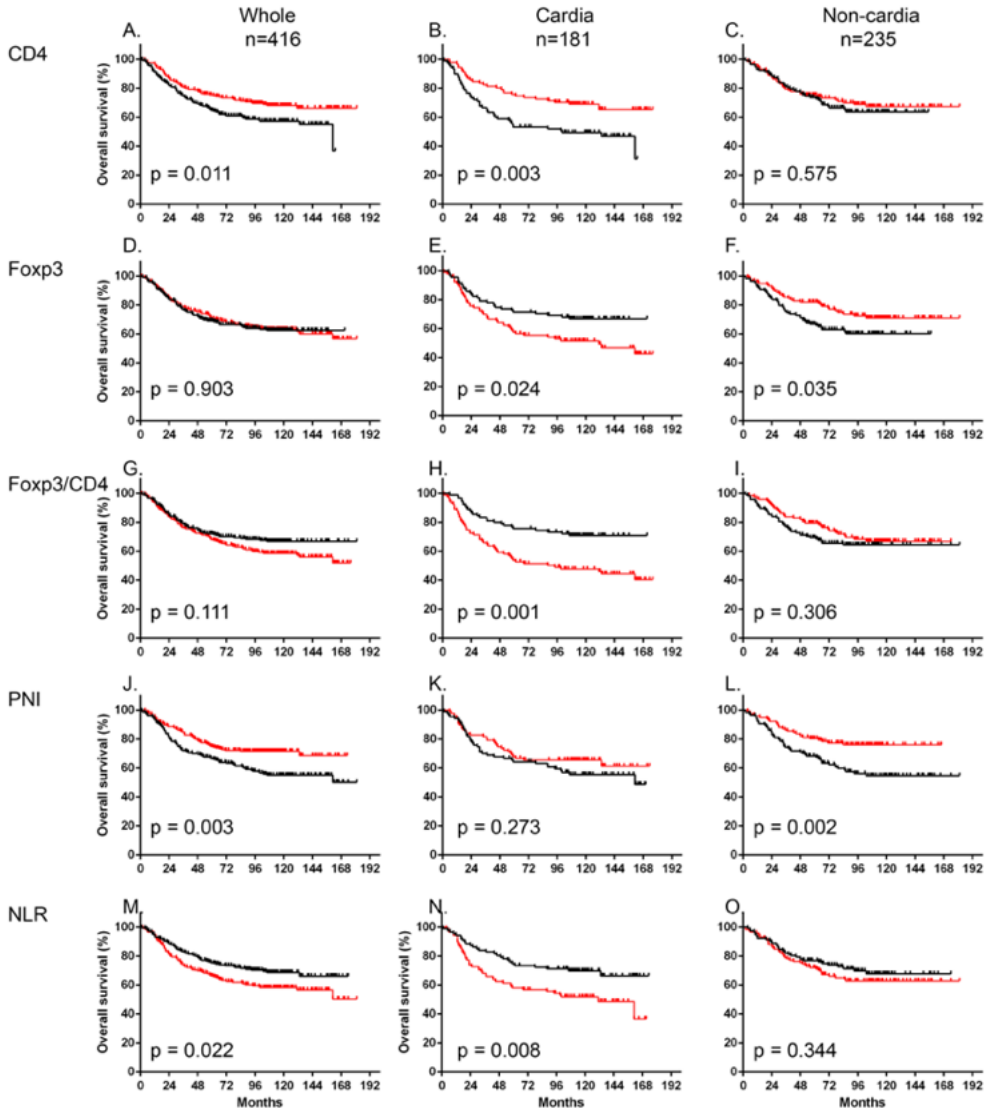

Figure 4. Survival according to local and systemic immune responses in cardia and non-cardia gastric cancer patients.

Kaplan-Meier analysis of overall survival in whole, cardia, and non-cardia cohorts for CD4 (**A-C**), Foxp3 (**D-F**), Foxp3/CD4 (**G-I**), PNI (**J-L**), and NLR (**M-O**) according to higher (red) and lower (black) levels than the median. This subset of TILs showed different prognostic impacts in cardia and non-cardia gastric adenocarcinoma patients. The median values of CD3, CD4, CD8, Foxp3, and granzyme B were as follows: CD3 (162.8), CD4 (87.5), CD8 (73.6), Foxp3 (15.6), and granzyme B (15.6), respectively.

Table 3. Univariate and multivariate analysis of factors associated with overall survival.

	Univariate			Multivariate		
	HR	95% CI	p-value	HR	95% CI	p-value
Whole cohort						
Age	1.001	0.988-1.013	0.905			
Sex	1.092	0.785-1.521	0.600			
Total gastrectomy	1.813	1.255-2.618	0.002			
Tumor size 40mm	2.387	1.695-3.362	<0.001			
T classification (T34/T12)	3.436	2.278-5.181	<0.001	2.158	1.385-3.363	0.001
N positive vs negative	3.136	2.195-4.480	<0.001	2.274	1.549-3.337	<0.001
CD3	0.998	0.996-1.001	0.147			
CD4	0.997	0.993-1.000	0.047			
CD8	0.997	0.993-1.001	0.175			
Foxp3	1.006	0.996-1.016	0.228			
Granzyme B	1.002	0.993-1.010	0.711			
Foxp3/CD4	1.008	1.002-1.013	0.006			
PNI	0.939	0.915-0.964	<0.001	0.953	0.928-0.978	<0.001
NLR	1.038	0.938-1.148	0.473			
Cardia						
Age	1.006	0.988-1.023	0.543			
Sex	1.002	0.618-1.627	0.992			
Total gastrectomy	N/A					
Tumor size 40mm	1.985	1.23-3.206	0.005			
T classification (T34/T12)	3.091	1.667-5.733	<0.001			
N positive vs negative	3.475	2.023-5.969	<0.001	3.347	1.912-5.858	<0.001
CD3	0.998	0.995-1.001	0.144			
CD4	0.996	0.992-1.000	0.028			
CD8	0.997	0.991-1.003	0.306			
Foxp3	1.011	1.001-1.021	0.039			
Granzyme B	0.998	0.988-1.008	0.704			
Foxp3/CD4	1.009	1.003-1.014	0.002	1.008	1.001-1.015	0.019
PNI	0.952	0.915-0.990	0.014			
NLR	1.058	0.899-1.246	0.498			
Non-cardia						
Age	0.996	0.979-1.014	0.666			
Sex	1.237	0.783-1.956	0.362			
Total gastrectomy	1.974	1.258-3.096	0.003	1.596	1.010-2.525	0.045
Tumor size 40mm	2.831	1.735-4.621	<0.001			
T classification (T34/T12)	3.642	2.097-6.325	<0.001	2.640	1.456-4.785	0.001
N positive vs negative	2.732	1.691-4.413	<0.001	1.781	1.065-2.980	0.028
CD3	0.999	0.995-1.004	0.736			
CD4	0.999	0.993-1.005	0.726			
CD8	0.998	0.992-1.004	0.579			
Foxp3	0.973	0.948-0.999	0.041	0.968	0.943-0.994	0.017
Granzyme B	0.981	0.951-1.013	0.241			
Foxp3/CD4	0.980	0.958-1.003	0.090			
PNI	0.929	0.897-0.963	<0.001	0.935	0.900-0.972	0.001
NLR	1.036	0.910-1.179	0.597			

A forward stepwise elimination with a threshold of p = 0.10 was used to select.
HR=hazard ratio; CI=Confidence interval; N/A not applicable
Abbreviations: Foxp3 = forkhead/winged helix transcription factor, NLR = neutrophil-to-lymphocyte ratio, PNI = prognostic nutritional index

Table 4. Univariate and multivariate analysis of factors associated with recurrence free survival.

	Univariate			Multivariate		
	HR	95% CI	p-value	HR	95% CI	p-value
Whole cohort						
Age	0.981	0.969-0.993	0.002			
Sex	1.394	1.007-1.929	0.046			
Total gastrectomy	1.602	1.122-2.289	0.010			
Tumor size 40mm	2.980	2.085-4.259	<0.001			
T classification (T34/T12)	5.770	3.563-9.344	<0.001	3.451	2.067-5.759	<0.001
N positive vs negative	5.929	3.903-9.005	<0.001	3.883	2.484-6.069	<0.001
CD3	0.998	0.996-1.001	0.177			
CD4	0.997	0.993-1.000	0.060			
CD8	0.996	0.991-1.000	0.052	0.994	0.989-0.999	0.011
Foxp3	1.002	0.992-1.013	0.670			
Granzyme B	1.000	0.991-1.009	0.992			
Foxp3/CD4	1.006	1.000-1.012	0.050			
PNI	0.955	0.931-0.980	0.001	0.974	0.949-1.000	0.048
NLR	1.033	0.933-1.145	0.531			
Cardia						
Age	0.991	0.973-1.009	0.317			
Sex	0.932	0.563-1.542	0.784			
Total gastrectomy	N/A					
Tumor size 40mm	3.484	1.994-6.087	<0.001			
T classification (T34/T12)	6.558	2.839-15.151	<0.001	2.788	1.131-6.867	0.026
N positive vs negative	7.512	3.726-15.144	<0.001	5.273	2.381-11.678	<0.001
CD3	0.997	0.994-1.000	0.090			
CD4	0.995	0.991-0.999	0.026			
CD8	0.996	0.990-1.003	0.236			
Foxp3	1.011	1.000-1.021	0.049			
Granzyme B	0.996	0.985-1.007	0.450			
Foxp3/CD4	1.009	1.003-1.014	0.003			
PNI	0.960	0.923-0.999	0.043			
NLR	1.050	0.885-1.245	0.575			
Non-cardia						
Age	0.972	0.956-0.988	0.001	0.983	0.98-1.000	0.044
Sex	2.022	1.300-3.146	0.002			
Total gastrectomy	1.785	1.146-2.779	0.010			
Tumor size 40mm	2.580	1.612-4.131	<0.001			
T classification (T34/T12)	5.362	2.953-9.734	<0.001	3.642	1.939-6.842	<0.001
N positive vs negative	5.202	3.034-8.920	<0.001	3.464	1.978-6.064	<0.001
CD3	0.999	0.996-1.004	0.952			
CD4	0.999	0.994-1.005	0.805			
CD8	0.995	0.989-1.002	0.158			
Foxp3	0.958	0.932-0.985	0.003			
Granzyme B	0.978	0.946-1.010	0.174			
Foxp3/CD4	0.966	0.942-0.991	0.009	0.960	0.934-0.987	0.004
PNI	0.953	0.920-0.986	0.006	0.946	0.910-0.983	0.005
NLR	1.027	0.902-1.169	0.691			

A forward stepwise elimination with a threshold of p = 0.10 was used to select.
HR=hazard ratio; CI=Confidence interval; N/A not applicable
Abbreviations: Foxp3 = forkhead/winged helix transcription factor, NLR = neutrophil-to-lymphocyte ratio, PNI = prognostic nutritional index

DISCUSSION

This study demonstrates that the prognostic significance of local and systemic immune responses significantly differs according to the location of gastric tumors. In cardia cancer, high Foxp3/CD4 ratio was a poor prognostic factor, whereas in non-cardia cancer, high Foxp3 counts and a high PNI value were favorable prognostic factors. The most significant discovery of this study was that the distribution and prognostic impact of Foxp3 differs between cardia and non-cardia cancers. In addition, PNI value was found to be an independent prognostic factor only in non-cardia cancers.

Previously, the prognostic impact of the Foxp3 subset of TILS in gastric cancer had conflicting conclusions, including being a good prognostic factor,(21-23) poor prognostic factor,(11-19) or having no prognostic impact.(24-27, 96) These studies differed in respect to country, tumor location, and histology. Furthermore, only a few studies were stage-adjusted for survival analysis. This has left unanswered questions as to whether the prognostic impact of TILs is associated with the clinicopathologic characteristics that are critical for the analysis of prognosis. In this study, it was hypothesized that stage and location information should be integrated into the interpretation of prognostic data.

In colorectal cancer, the anatomical site of the tumor is an important factor for clinical management.(37, 38) Left and right colon cancers have different clinicopathological characteristics: for example, bacteria increase in number with a positive gradient from the proximal to the distal colon.(97) In addition, a study that surveyed the linear distribution of immune cells showed decreasing numbers of CD3 cells and increasing numbers of CD8 cells when moving from the ascending colon to the rectum.(98) Moreover, the incidence of right colon cancer has recently increased, with a simultaneous fall in the incidence of left colon cancer.(99) Furthermore, increasing evidence has revealed distinct

differences in clinical and molecular pathway characteristics in association with the anatomical site of the tumor.(39, 40) Thus, colorectal cancer is no longer regarded as a single entity.

A phenomenon, that the different anatomical locations and functions of each tissue are also affected by different tumor microenvironments, may be applicable to gastric cancer. The increasing incidence of cardia cancers and concurrent decreasing incidence of non-cardia cancers is a worldwide phenomenon.(41, 46-49) Recent epidemiologic studies have suggested that negative associations between gastric cancer and cardia cancer in both incidence and time are caused by a common environmental factor that predisposes an individual to one and protects from the other:(46, 49) these studies suggested reduced acidity as a cause of non-cardia cancer and high acidity as a cause of cardia cancer. Although no conclusive answer with regards to why regulatory T cells had opposite prognostic impacts in cardia and non-cardia cancer was presented, the studies suggested that cancers of cardia and non-cardia origin do not share similar carcinogenesis and progression mechanisms.

The present study identified PNI as an independent prognostic factor only in non-cardia cancer. One benefit of PNI and NLRs is that, contract to TILs, which are clinically sampled once during surgery, PNI and NLR values are easy to acquire repeatedly in clinical settings.(29) Most studies reporting the prognostic impact of PNI have utilized cohorts from East Asia, where most gastric cancers are non-cardia cancers.(28-30) One report from China showed that PNI was not an independent factor for survival of cardia cancer patients.(100) These results support the result of current study in that PNI is not associated with prognosis in cardia cancer, but is prognostic in non-cardia cancer.

To the best of my knowledge, no investigation into the relationship between TILs and PNI or NLR have been conducted in gastric cancer. The systemic inflammatory response is presumed to upregulate mediators of innate immunity, which promotes tumor progression.(101) Thus, the secondary aim of the study was to identify whether systemic immune responses, reflected by PNI or NLR, can represent local immune responses from TILs. Based on the findings of this study, PNI and NLR may not serve as a surrogate marker of the micro-immune environment like TILs.

The present study has limitations. First, based on the nature of retrospective studies, there was a possible bias during patient selection. Second, this study did not evaluate the status of *H. pylori*, which could be a confounding factor as it is associated with inflammation. Despite these limitations, the strength of the study is that it evaluated prognosis in association with stage and other clinicopathological factors that affect survival.(102) It was revealed that the prognostic value of TILs and PNI differ depending on the location within the stomach. In addition, it investigated the association of systemic and local immune responses using the parameters of PNI, NLR, and subsets of TILs. Therefore, this study revealed the need for future studies. For example, a study on the association of PD-L1 or PD-1 expression with TILs according to the location of tumor in the stomach would be useful for checkpoint block therapy.

In summary, this study highlights the importance of tumor location in association with local and systemic immune responses in gastric cancer patients. Cardia cancer is different from non-cardia cancer in terms of the impact of local and systemic immune responses on prognosis. Regulatory T lymphocytes was associated with an unfavorable prognosis in cardia and a favorable prognosis in non-cardia cancer.

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국문초록

서론: 종양침윤림프구와 예후영양지수는 각각 국소와 전신 면역상태를 나타내는 지표로 연구되어 왔으나 위암에서의 의미는 아직 명확히 밝혀지지 않았다. 이 연구의 목적은 위암의 위치에 따른 면역반응의 차이를 이 두 지표의 변화를 통하여 연구하는 것이다.

방법: 2001년-2010년 사이 시행된 416례의 위암 절제술을 시행한 환자를 대상으로 CD3, CD4, CD8, Foxp3, granzyme B 에 대한 일차 항체로 염색을 시행 후 면역화학염색을 시행하였다. 예후영양지수는 7781명의 위암 절제술을 시행한 환자의 수술 전 혈중 알부민과 림프구의 숫자를 이용하여 계산하였다.

결과: 분문부는 비분문부에 비해 낮은 CD8과 높은 Foxp3, granzyme B 숫자를 보였고 PNI와 NLR의 차이는 없었다. 다변량 분석결과 분문부에서 임파선 전이, 높은 Foxp3/CD4분율은 나쁜 예후 인자였고 비분문부에서는 전체 절제술, 진행된 T병기, 임파선 전이, 낮은 Foxp3, 낮은 예후영양지수가 나쁜 예후 인자였다.

결론: 예후영양지수와 종양침윤윤림프구의 분포는 위암의 위에서의 위치에 따라 다른 예후 인자자로서의 의미를 가지고 특히 조절 T 림프구는 분문부에서는 나쁜 예후 인자 비분문부에서는 좋은 예후인자로서의 의미를 가진다.

주요어: 종양침윤 림프구; 조절 T-림프구; 예후영양지수; 분문부암; 위암; 예후; 면역반응

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